RAPID COMMUNICATION

A Missense Mutation in γ-Glutamyl Carboxylase Gene Causes Combined Deficiency of All Vitamin K-Dependent Blood Coagulation Factors

By Benjamin Brenner, Beatriz Sánchez-Vega, Sheue-Mei Wu, Naomi Lanir, Darrel W. Stafford, and Jesus Solera

To identify potential mutations in the γ-glutamyl carboxylase gene, the sequence of all exons and intron/exon borders was determined in 4 patients from a consanguineous kindred with combined deficiency of all vitamin K-dependent procoagulants and anticoagulants and resulted in the conversion of an arginine codon (CTG) to leucine codon (CCG) at residue 394. Screening of this mutation based on introduction of Alu I site in amplified fragment from normal allele but not from the mutated allele showed that 13 asymptomatic members of the kindred were heterozygous for the mutation. The mutation was not found in 340 unrelated normal chromosomes. The segregation pattern of the mutation which is the first reported in the γ-glutamyl carboxylase gene fits perfectly with phenotype of the disorder and confirms the suggested autosomal recessive pattern of inheritance of combined deficiency of all vitamin K-dependent procoagulants and anticoagulants in this kindred. The mutated carboxylase protein expressed in Dro sophila cells was stable but demonstrated threefold reduced activity compared with WT carboxylase, confirming that the L394R mutation results in a defective carboxylase.

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VITAMIN K IS A NECESSARY cofactor for the hepatic carboxylation of glutamic acid residues in a number of proteins, including the procoagulants factors II, VII, IX, and X; the anticoagulants protein C and protein S; and other proteins such as osteocalcin and matrix Gla protein. This carboxylation is required for normal hemostasis, because it enables calcium binding and attachment of the procoagulants and anticoagulants to phospholipids.1,2

γ-Glutamyl carboxylase is an integral membrane microsomal enzyme located in the rough endoplasmic reticulum. It carboxylates glutamate residues located in the Gla domain of vitamin K-dependent coagulation factors.3,4 The carboxylation reaction is dependent on reduced vitamin K (KH2), which is converted to vitamin K epoxide during carboxylation, and must be regenerated by the vitamin K epoxide reductase for carboxylation to continue.5

Hereditary combined deficiency of vitamin K-dependent procoagulants is a rare bleeding disorder that has been reported in only a few patients.6-13 Deficiency of the anticoagulants protein C and protein S has been reported in some of these patients.12,13 Theoretically, this disorder may stem from functional deficiency of either the γ-glutamyl carboxylase or the vitamin K epoxide reductase.

We have previously reported an offspring of consanguinous marriage in a kindred with hereditary deficiency of all vitamin K-dependent procoagulants and anticoagulants.12 Normal epoxide reductase function was demonstrated by undetectable vitamin K epoxide serum levels. Impairment of Glaphospholipid time (APTT) was longer than 180 seconds. No response to 1 mg protein C activity was assayed by a one-stage coagulation assay.12 Protein S:Ag (PS:Ag) was analyzed by electroimmunoassay, using the following antibody solutions. Tris Tricine (0.08 mol/L Tris and 0.02 mol/L Tricine) containing 0.2% goat anti-protein S antibodies that recognize total protein S. A 1% agarose (Seakem; FMC Bioproducts, Rockland, ME) was used in all electroimmunoassays and gels were run at room temperature. Polyclonal antibodies used were commercial (Stago, Asnieres, France). Protein C activity was assayed by chromogenic substrate (Stachrom protein C; Stago). Normal range for each assay was determined by studying 30 normal individuals.

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MATERIALS AND METHODS

Blood collection. After approval of informed consent, citrated blood samples were obtained for coagulation assays and EDTA samples were obtained for DNA analysis.

Coagulation assays. Factors II, VII, IX, and X activities were assayed by a one-stage coagulation assay.12 Protein S:Ag (PS:Ag) was analyzed by electroimmunoassay, using the following antibody solutions. Tris Tricine (0.08 mol/L Tris and 0.02 mol/L Tricine) containing 0.2% goat anti-protein S antibodies that recognize total protein S. A 1% agarose (Seakem; FMC Bioproducts, Rockland, ME) was used in all electroimmunoassays and gels were run at room temperature. Polyclonal antibodies used were commercial (Stago, Asnieres, France). Protein C activity was assayed by chromogenic substrate (Stachrom protein C; Stago). Normal range for each assay was determined by studying 30 normal individuals.

Case reports. Patient no. 20, the 10th female offspring of consanguinous asymptomatic parents of an Arab origin, presented in 1982 shortly after birth with multiple ecchymoses and bleeding from puncture sites (Fig 1). An older sibling (hatched symbol) had died in infancy from uncontrollable umbilical bleeding. The prothrombin time (PT) of patient no. 20 was longer than 120 seconds, and the activated partial thromboplastin time (aPTT) was longer than 180 seconds. No response to 1 mg
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Fig 1. Pedigree of G family showing the segregation of the mutation. Dashed lines indicate undefined number of generations. Haplotypes are built with L394R mutation and intragenic polymorphisms. Only haplotypes of members of the family available for the study are shown. Affected subjects, carrier subjects, and unaffected subjects are indicated by solid symbols, half-solid symbols, and open symbols, respectively. The member indicated with a hatched symbol died of bleeding.

Table 1. Vitamin K-Dependent Procoagulants and Anticoagulants Plasma Levels

<table>
<thead>
<tr>
<th>Patient</th>
<th>II:C</th>
<th>VII:C</th>
<th>IX:C</th>
<th>X:C</th>
<th>PC act</th>
<th>PS:Ag</th>
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<tbody>
<tr>
<td>3</td>
<td>107</td>
<td>140</td>
<td>92</td>
<td>83</td>
<td>ND</td>
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<td>4</td>
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<td>1</td>
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<td>114</td>
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<td>ND</td>
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<td>2</td>
<td>107</td>
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<td>113</td>
<td>100</td>
<td>ND</td>
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<tr>
<td>7</td>
<td>107</td>
<td>90</td>
<td>96</td>
<td>84</td>
<td>107</td>
<td>95</td>
</tr>
<tr>
<td>17</td>
<td>116</td>
<td>79</td>
<td>68</td>
<td>89</td>
<td>120</td>
<td>65</td>
</tr>
<tr>
<td>20*</td>
<td>18</td>
<td>25</td>
<td>37</td>
<td>15</td>
<td>45</td>
<td>34</td>
</tr>
<tr>
<td>21*</td>
<td>45</td>
<td>43</td>
<td>89</td>
<td>27</td>
<td>73</td>
<td>35</td>
</tr>
<tr>
<td>22*</td>
<td>51</td>
<td>23</td>
<td>55</td>
<td>17</td>
<td>84</td>
<td>28</td>
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<td>23*</td>
<td>24</td>
<td>47</td>
<td>33</td>
<td>16</td>
<td>71</td>
<td>57</td>
</tr>
</tbody>
</table>

Normal range 77-125 63-139 63-155 55-160 65-146 74-126

Values are units per deciliter.
Abbreviation: ND, not done.
*Levels obtained on chronic weekly subcutaneous 10 mg vitamin K therapy.
Polymerase chain reaction (PCR) amplification and direct sequencing of exons. The 15 exons and intron/exon flanking sequences of the γ-glutamyl carboxylase gene were screened for mutations. All functionally important fragments of the gene were included and both strands were sequenced. The screening consisted of PCR amplification and further direct sequencing of amplified products using a commercial kit, according to the manufacturer’s instructions (Amersham, Arlington Heights, IL). The primers and PCR conditions for each exon are collected in Table 2.

Analysis of L394R mutation. We designed a specific PCR approach for the analysis of L394R mutation. First, we amplified a 810-bp fragment with primers E-I/5 and E-X/3 (Table 2), including exons 9 and 10. The reaction mixture contained in a volume of 25 µL the following: 50 ng of genomic DNA, 400 ng of each primer, 200 µmol/L dNTPs, 1.5 mmol/L MgCl₂, 2.5 µL of 10× buffer, and 1 U of Taq DNA polymerase (Boehringer Mannheim). Amplification was performed with 30 cycles of the following profile: denaturation at 94°C for 1 minute, annealing at 55°C for 45 seconds, and extension at 72°C for 5 seconds.

The PCR product was digested with Alu I and subjected to electrophoresis in 4% agarose.

Expression studies. Normal and mutant HGC were expressed in Drosophila cells using the metallothionein promoter. DNA containing the human γ-glutamyl carboxylase cDNA and the cytochrome P-450-resistance gene were cotransfected into S2 Drosophila cells with calcium chloride. Positive clones were selected with hygromycin at 150 µg/mL. For expression, the metallothionein promoter was induced with 500 µmol/L copper sulphate when the cell density had reached about 5 million cells/mL. Twenty-four hours after induction, the cells were harvested, concentrated and mixed with a cocktail of protease inhibitors. For carboxylase assays, 3 million cells in 35 µL were lysed with 1.4% CHAPS/phosphatidyl choline at 10 mmol/L MOPS, pH 7.5, and 700 µmol/L NaCl on ice for 20 minutes. Reaction was performed at a total volume of 125 µL with 1.2 mmol/L FLEEL, 16 µmol/L proFIX, 820 µmol/L ammonium sulfate, 222 µmol/L reduced vitamin K, and 1.4 mmol/L CO₂ and incubated at 25°C for 30 minutes. The samples were processed as previously described. For estimation of the relative amounts of normal and mutant carboxylase, samples were Western blotted and probed with an antibody. The first, RGS.His (Qiagen, Valencia, CA) was followed by a peroxidase-conjugated goat antirabbit antibody (Jackson Laboratories, West Grove, PA), and the bands were visualized with Amersham’s ECL reagent.

Table 2. PCR Amplifications and Direct Sequencing of γ-Glutamyl Carboxylase Gene

<table>
<thead>
<tr>
<th>Exon No.</th>
<th>PCR Conditions</th>
<th>No. of Cycles</th>
<th>Primer</th>
<th>Sequence, 5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon I</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-I/5</td>
<td>CTA GGG AGA CAA ATT CCT CTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E-I/3.2</td>
<td>ACC GGG AGA CAC TGG CGT C</td>
</tr>
<tr>
<td>Exon II</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-II/5</td>
<td>GAG CTG TTG CGG TGA TTT CT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E-II/3</td>
<td>AGA GAT CCT CAT TCT CCA CCC T</td>
</tr>
<tr>
<td>Exon III</td>
<td>94°C/45 s 56°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-III/3</td>
<td>GTT CTT TGA TGG TGC TAG</td>
</tr>
<tr>
<td>Exon IV</td>
<td>94°C/45 s 55°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-IV/3</td>
<td>TTA CCA CCA TGG CCA AGC CA</td>
</tr>
<tr>
<td>Exon V</td>
<td>94°C/45 s 49°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-V/3</td>
<td>CCC CCC AAT GGT TAC ATC CT</td>
</tr>
<tr>
<td>Exon VI</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-VI/3</td>
<td>CTA TAC TGA GAG GAG CAG TCA CTT</td>
</tr>
<tr>
<td>Exon VII</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-VII/5.2</td>
<td>TGG GTG CTG TGA TGG TGC TAG A</td>
</tr>
<tr>
<td>Exon VIII</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-VIII/5</td>
<td>AGA CCC AGC CAA ACT CCT</td>
</tr>
<tr>
<td>Exon IX</td>
<td>94°C/45 s 58°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-IX/3</td>
<td>ATG TCT GAT GCC AAC</td>
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<tr>
<td>Exon X</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-I/5</td>
<td>CTG GTG TTG CAG CCC CTT CTT 1370C</td>
</tr>
<tr>
<td>Exon XI</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-X/3</td>
<td>CAG AAG GGG CTC TGG TAC CCG</td>
</tr>
<tr>
<td>Exon XII</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-XI/5.4</td>
<td>GGT GGC TGT GATU TCC TTA GAA</td>
</tr>
<tr>
<td>Exon XIII</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-XII/5.4</td>
<td>GCC ATG GGG TGG GAT GAA C</td>
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<tr>
<td>Exon XIV</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-XIII/5.2</td>
<td>TGG GGC CAT AAG CTC TGG GCT AGA</td>
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<tr>
<td>Exon XV</td>
<td>94°C/45 s 57°C/30 s 72°C/1 min</td>
<td>30</td>
<td>E-XV/5</td>
<td>CTA GCT GGC AGA AGA GGA GCT</td>
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<td></td>
<td>E-XV/3</td>
<td>TGT CCA TTG CAT AGA ATG GCT</td>
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RESULTS

Identification of the mutation. To identify potential mutations in the γ-glutamyl carboxylase gene, the sequence of all exons and intron/exon borders was determined and the results were compared with the normal genomic sequence.16 The results of this analysis that were preliminarily reported at the ISTH Florence meeting18 showed that all patients were homozygous for a point mutation in exon 9, which resulted in the conversion of an arginine codon (CTG) to the leucine codon (CGG) at residue 394 (Fig 2A and B). There were no other nucleotide changes that would lead to an amino acid substitution. Exon 9 codes for a carboxylase domain showing some sequence similarity to cytochrome b and is completely conserved in human and bovine carboxylase. We have designed a PCR strategy for the screening of this mutation based on the introduction of an Alu I site in the amplified fragment from the normal allele but not from the mutated allele (Fig 2C). With this approach, 340 unrelated normal chromosomes were analyzed and the mutation was not found in any case. This PCR strategy along with haplotype analysis of the 4 known intragenic polymorphisms in the γ-glutamyl carboxylase gene was used in every member of the pedigree and confirmed 6 normal siblings, 13 heterozygotes, and the 4 homozygotes for the L394R mutation in the kindred (Fig 1). Carriers of the mutation showed no clinical or analytical alteration.

Expression studies. To address the question of whether the expressed, mutated protein was stable and was present in our assays in amounts similar to normal carboxylase, normal and mutant carboxylase were expressed in Drosophila cells.18 Both constructs had a histidine tag at their amino terminus, which had no effect on carboxylase activity. Drosophila cells are free of endogenous carboxylase activity and are therefore appropriate for comparison of carboxylase activity. Figure 3 shows that approximately equivalent amounts of carboxylase were present in extracts from normal and L394R carboxylase preparations. Table 3 demonstrates that the mutation of leucine 394 to arginine results in an at least threefold reduction in carboxylase activity and demonstrates that the cause of the defect is truly carboxylase related.

DISCUSSION

The segregation pattern of the L394R mutation fits perfectly with the phenotype of the disease and confirms the suggested autosomal recessive pattern of inheritance for combined deficiency of the vitamin K-dependent coagulation factors in this family. Haplotype analysis provided further proof for the common origin of both alleles of the L394R mutation in affected patients.

Reported cases of mild or moderate combined deficiency of vitamin K-dependent procoagulants were usually diagnosed at an older age, when the patients presented with mucocutaneous or postsurgical bleeding.8-10 Most reports concern isolated cases with deficiency of all vitamin K-dependent coagulation factors.

The markedly low levels of vitamin K-dependent coagulation factors in patient no. 20 who presented as a neonate could partly result from the added effect of immaturity of neonatal liver.

Expression studies demonstrated at least threefold reduced activity of the L394R γ-glutamyl carboxylase. This fits nicely with the detectable plasma procoagulants levels at diagnosis and may explain why the L394R mutation that results in

Fig 2. Identification of L394R mutation. (A) Schematic representation of human γ-glutamyl carboxylase gene structure showing the situation of exons. In detail is a fragment of exon 9 sequence containing the nucleotide substitution at codon 394. The transversion T to G (underlined) at that position causes a Leucine to Arginine replacement in the protein. (B) Direct sequencing of the genomic DNA from one patient depicts homozygosity for the mutation. (C) Analysis of L394R mutation by PCR. Electrophoresis of amplified DNA using the mutated oligonucleotide designed to introduce an Alu I restriction site in normal allele but not in mutant allele. Lane 1, normal control; lane 2, patient's DNA homozygous for the mutant allele; lane 3, heterozygous pattern.
moderate to severe reduction of vitamin K-dependent coagulation factors levels is viable, in contrast to mutations that result in total abrogation of γ-glutamyl carboxylase expression.

Weekly subcutaneous administration of 10 mg vitamin K resulted in an increase of procoagulants levels in all 4 subjects with γ-glutamyl carboxylase L394R mutation. Although the increase was more pronounced in factor IX:C levels and less in factor X:C levels, it was sufficient for achieving hemostatic levels. In fact, during 30 patient years on vitamin K therapy, no major bleeding and only rare minor bleeding episodes were observed. Interestingly, a previously reported 2 siblings responded with total correction of plasma procoagulant levels after parenteral administration of vitamin K.9

Recognition of the vitamin K-dependent coagulation factors by γ-glutamyl carboxylase is dependent on 18 amino acid propeptide at the N-terminal of the coagulation factor, which serves as a docking site for interaction with γ-glutamyl carboxylase.19-21 Site-directed mutagenesis studies suggest that regions around residues 234, 406, and 513 define in part the propeptide binding site.22 The L394R mutation is in proximity to a propeptide binding site on γ-glutamyl carboxylase,23 suggesting the possibility of reduced propeptide binding. Theoretically, the observed increase in coagulation factor levels after high-dose vitamin K administration may be explained by an increased affinity or by an overcome of a normal or reduced affinity of vitamin KH2 to the L394R γ-glutamyl carboxylase. However, γ-glutamyl carboxylase is a complicated enzyme with several substrates, and further experiments are required to elucidate the role of the L394R mutation on different aspects of carboxylation.

The enzymatic aspects of carboxylation have been characterized in Devon Rex cats and congenital deficiency of glutamyl carboxylase.24 Phenotypical expression of defective glutamyl carboxylase in affected cats is similar to the clinical phenotype of the L394R mutation. In that study, kinetic parameters showed a potential impaired recognition of the propeptide sequence in nascent vitamin K coagulation polypeptides.24

The L394R mutation is the first reported naturally occurring mutation in the human γ-glutamyl carboxylase gene that is responsible for a combined deficiency of vitamin K-dependent coagulation factors. Identification of L394R mutation will allow future direct diagnosis of potential carriers of γ-glutamyl carboxylase deficiency. This will enable genetic counseling for a severe heritable bleeding disorder in this kindred with a high consanguinity rate.

ACKNOWLEDGMENT

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REFERENCES


Table 3. Constructs Activity of Wild-Type and L394R γ-Glutamyl Carboxylase

<table>
<thead>
<tr>
<th>Construct</th>
<th>Activity (cpm/h)</th>
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<tr>
<td>Wild-type</td>
<td>18,215 ± 78</td>
</tr>
<tr>
<td>L394R</td>
<td>5,900 ± 274</td>
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</table>
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