

## ORIGINAL ARTICLE

# Homozygous intronic mutation leading to inefficient transcription combined with a novel frameshift mutation in *F13A1* gene causes FXIII deficiency

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Two novel mutations, 602–605delAAAG in exon 5 and Int1(+12)C>A, of the *F13A1* gene were identified in a Chinese factor XIII (FXIII)-deficient family. The 602–605delAAAG mutation results in the premature termination of translation. To determine the functional effect of the Int1(+12)A mutation, we transiently expressed luciferase reporters in U937 cells. We found that the first 951 bp of *F13A1* intron 1 is involved in regulating the expression of the *F13A1* gene and that Int1(+12)A results in its reduced expression. Electrophoretic mobility shift assay indicated that Int1(+12)A causes reduced protein binding. An Sp1 site was predicted in the sequence containing Int1(+12)C, which the Int1(+12)A mutation eliminates. Co-transfection of a plasmid expressing Sp1 revealed that Sp1 is involved in regulating the expression of FXIII and that Int1(+12)A leads to inefficient transcription. These results provide the first insight into a novel regulatory mechanism involving intron 1 in the *F13A1* gene.

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## INTRODUCTION

Inherited deficiency of FXIII is a rare, autosomal recessive disorder, leading to life-long bleeding diathesis and defective wound healing in affected patients, and a high risk of spontaneous abortion in affected women.<sup>1–2</sup> Mutations in the A or B subunit gene are responsible for the disorder.<sup>1–7</sup> The disorder usually results from mutations in subunit A, but is occasionally attributable to mutations in subunit B (<http://www.hgmd.cf.ac.uk/> and <http://www.fl3-database.de>). We describe two novel mutations in a Chinese FXIII-deficient family and characterize a novel mechanism involved in regulating the expression of the *F13A1* gene.

## MATERIALS AND METHODS

The proband was female, born in 1983 at full term following a normal delivery. She had umbilical cord bleeding a few days after birth and recurrent posttraumatic subcutaneous bleeding episodes. Her younger brother displayed a slight bleeding disorder. Other family members had no bleeding tendency. The proband was diagnosed using a clot stability test in 5 M urea. Informed consent was obtained from all family members in accordance with the Declaration of Helsinki, and the study was approved by the ethical review committee of the Affiliated Hospital of Hainan Medical College. FXIII activity and plasma FXIII antigen were determined as described previously, using fibrinogen

and 5-biotinamido)pentylamine as substrates<sup>8</sup> and an enzyme-linked immunosorbent assay, respectively (Supplementary materials).

Genomic DNA was extracted from leukocytes. Exons 1–15 of the *F13A1* gene were amplified by PCR (Supplementary Table 1). Mutations were screened by single-stranded conformation polymorphism analysis and confirmed by DNA sequencing. Total RNA was extracted from lymphocytes and reverse transcribed with the SuperScript II reverse transcription (RT) kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The complementary DNA generated was amplified, using  $\beta$ -actin as the internal control.

A PCR-based strategy was used to generate constructs containing 114 bp of the minimum promoter,<sup>9</sup> exon 1 and 3' serial deletions of intron 1 from nt +951 (Figure 1a and Supplementary materials). The Int1+12A mutation was introduced into the construct using site-directed mutagenesis. The sequences of all reporter constructs were verified by DNA sequencing. The constructs and pGL3Basic, used as the negative control, were transfected into cultured cells with Lipofectamine 2000 (Invitrogen). The cells were harvested 48 h after transfection and cell lysates were prepared. Reporter gene activities were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Luciferase intensities were normalized to the internal control (SV40–Renilla) to assess transfection efficiency. All experiments were repeated at least three times using three different preparations of each construct tested. Plasmid pN3-Sp1 (Invitrogen), which expresses Sp1, was co-transfected into U937 cells with a construct with or without the Int1+12A mutation.

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Nuclear extracts were prepared from U937 cells. Double-stranded oligonucleotide probes (Supplementary Table 2), binding the region from -4 to +26 bp, with nucleotide C or A at position +12 in intron 1, were end-labeled with  $\gamma$ -<sup>32</sup>P-ATP. Binding reactions were performed as described in Supplementary materials. In competition experiments, a 125-fold molar excess of unlabeled probe was included. The DNA-protein complexes were separated on 8% polyacrylamide gels, which were dried under vacuum and exposed to X-ray film.

## RESULTS AND DISCUSSION

Assays of FXIII activity and plasma FXIIIa antigen in the patients and family members revealed very low relative levels of FXIII activity and FXIIIa antigen in the patients (Supplementary Table 3). These data confirmed the previous diagnosis of FXIII deficiency made with the urea solubility test.

Single-stranded conformation polymorphism analysis revealed band shifts in the amplification products of exon 1 (Supplementary Figure 1) and exon 5 (Supplementary Figure 1), indicating mutations in these exons. No abnormal band shift was observed in 120 healthy individuals, confirming the band shifts in exons 1 and 5 of the patients as causative. Therefore, the abnormal band shifts in exons 1 and 5 are probably associated with the patient's FXIII deficiency.

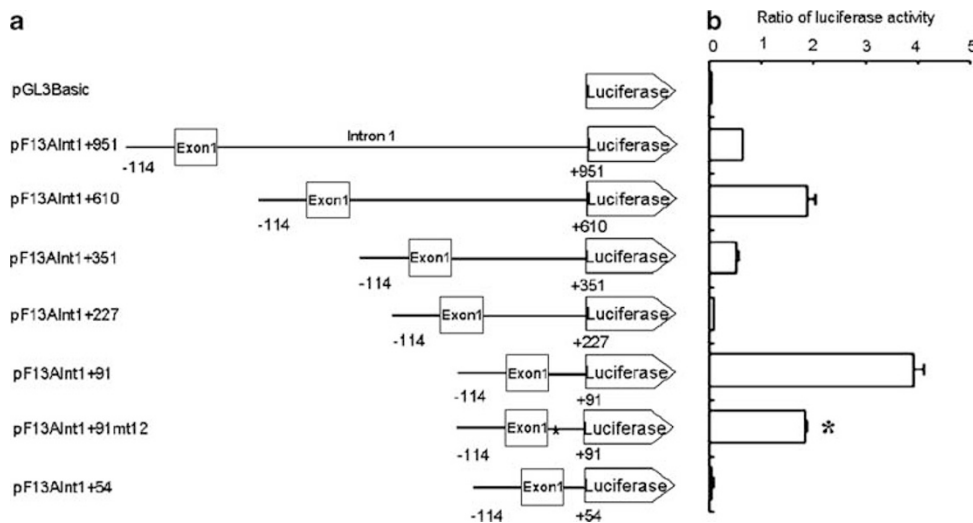
Sequencing analysis revealed that the band shift in exon 1 resulted from a C-to-A transversion at position +12 in intron 1 (Int1(+12)C>A). The proband and her younger brother were homozygous for the Int1(+12)C>A mutation, whereas her mother and father were heterozygous for the Int1(+12)C>A mutation (Supplementary Figure 2). However, the variation is polymorphic in African and European descents (<http://pga.gs.washington.edu>), which may be due to racial differences. Indeed, difference in allelic frequency, such as F13A Val34Leu polymorphism, had been observed between Asian and other populations.<sup>10,11</sup>

Direct sequencing of the exon 5 PCR fragment revealed two separate sequences (Supplementary Figure 3), suggesting a deletion or insertion in one of the alleles. Subcloning and sequencing revealed a deletion of AAAG at 602–605 (602–605delAAAG) or AAGA at 603–606 in the GAGAAAGAAAGAGAAGAG region, leading to a

predicted truncated peptide of 204 amino acids (Supplementary Figure 3). There are no other mutations in *F13A1* gene. These results confirm that the two mutations are responsible for the FXIII deficiency in the patients. A pedigree analysis of the family is shown in Supplementary Figure 4.

Previous studies of other genes have shown that intronic sequences are involved in regulating gene expression.<sup>12–16</sup> In this study, reduced levels of FXIIIa antigen and mRNA were observed in the patients (Supplementary Table 3 and Supplementary Figure 5), suggesting that the Int1(+12)C>A mutation affects the expression of the *F13A1* gene. To determine the functional effects of the Int1(+12)C>A mutation in the *F13A1* gene, luciferase reporters were transiently expressed in U937 cells. The minimal *F13A1* promoter plus exon 1 and the first 951 bp of intron 1 correctly transcribed a luciferase reporter gene (Figure 1b). Serial 3' deletion analysis revealed that deletions from +951 to +610, +351 and +91 resulted in increased luciferase activity, whereas deletions from +951 to +227 and +54 resulted in dramatically reduced luciferase activity (Figure 1b), suggesting that the first 951 bp of intron 1 are involved in regulating *F13A1* gene expression. Because the deletion from +951 to +91 (construct pF13AInt+91) displayed the highest luciferase activity, Int1(+12)A was introduced into pF13AInt+91 to evaluate its effect. The luciferase activity in cells transfected with mutant construct pF13AInt+91mt12 was significantly reduced compared with that in cells transfected with construct pF13AInt+91 (Figure 1b). This is consistent with the reduced FXIIIa antigen levels and reduced mRNA levels observed. These results strongly suggest that the Int1(+12)C>A mutation is a transcription-affecting mutation associated with FXIII deficiency.

Prediction of the relevant *cis* element in the first 951 bp of intron 1 of the *F13A1* gene with MatInspector (<http://www.genomatix.de>) identified several putative Sp1-binding sites in the intron 1 region (Figure 2a). The Int1(+12)A mutation eliminates the Sp1-binding site between base pairs +8 and +15 of intron 1, indicating that this site might be involved in transcription. An electrophoretic mobility shift assay revealed a single complex formed by both the wild-type probe and mutated probe. However, the band representing the complex formed with the mutated probe was markedly weaker (Figure 2b).



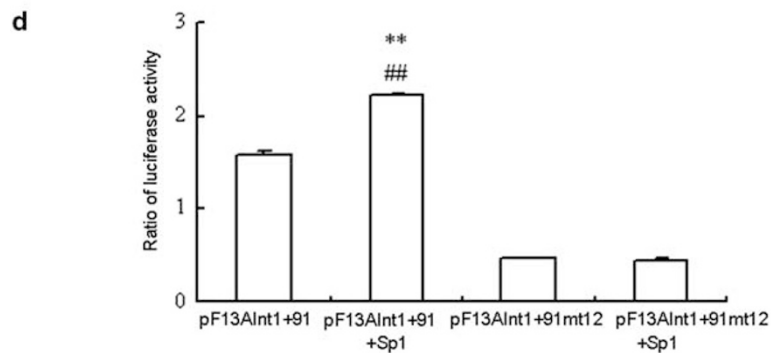
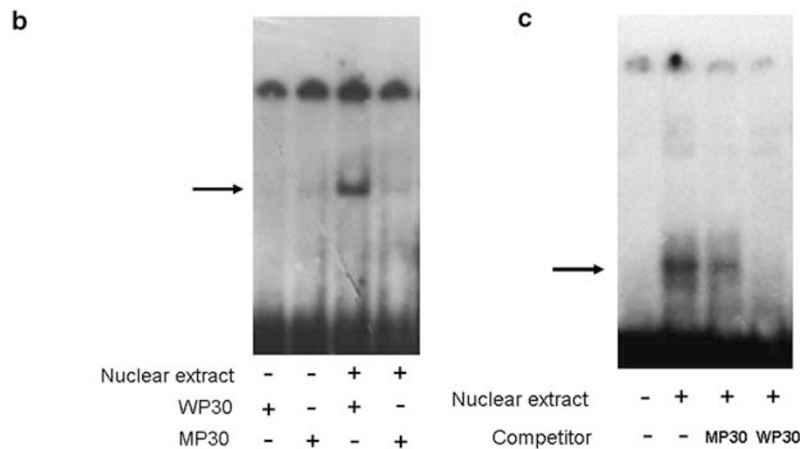
**Figure 1** Functional characterization of the sequence of intron 1 of the *F13A1* gene. (a) The diagram shows various fragments created by serial 3' deletions of the first 951 nucleotides in intron 1 of the *F13A1* gene, which were inserted into pGL3Basic to produce the constructs. (b) The relative luciferase activity expressed by each construct in U937 cells. \* Indicates a statistically significant difference ( $P < 0.01$ ) in the relative luciferase activity between the normal construct pF13AInt1+91 and the mutant construct pF13AInt1+91mt12.

**a**

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-114 CCCCTACTGG CTCCAGCTGT GGAGAAGGGG GAGGAGAAAA CCTGTGTTGGA CAGGGGGAGGA
-54 GGGTGAGGGC TCCTCTTAGG AAGTTATTTA AGAGCCAACT GTCTTGTTCTT TCCCGAGTCC
      *+1
+7 GTTTGAGGAA GTCCCCGAGG CGCACAGAGC AAGCCCACGC GAGGGCACCT CTGGAGGGGA
      Sp1 ↓
+67 GCGCCTGCAG]GTAAGCCACC GCCCTGCAC CCTATGAGCC AGGGCCCGCT GGTCCACCT
      ←
+127 TCTGCACCTC GGTTCCTGG TTGAACCAGC AAGCGGCTTG CTCTGGGCCC TGTGGGCGCC
      Sp1
+187 GTCACAGGCA GCTCCACTTG CCCAATCCTG GCTTCCC GCCA ACTCCG CACCTGCCCA
      Sp1
+247 GCCCAGCTTC TAAGCGGGAC TTCTCTCAGG CTCTCGAGGC AGCTCTCCC AAGCCGTTGC
      →
+307 TTGCTTAGAA TGCCACAAA TGGGGATGGG GGAGTTGGAG GTGCTTCCCC AAGGGACGAG
+367 CCCCCTTTCC TCAGGGAGCA GATTATGGAG CTGGAACACA AAAGTTGGAG GGAGTGGGGC
+427 CAAGTGAGAA CAGCCTGCTA GACTGGAACC CCGCCGGCTT GGCTCCCCAC TCCTGGCGGC
+487 CTCCCCACTC CTGCCATCG CCTGCCCTCG TCCCAATTTG GAGCTGAACT CGAGCCACTT
      SP1
+547 CTCTCCTTA GACCCGCCCA GAAGCGAGGG CTCTGCGCCG CCGCACTCAC CCAGCCAGGC
      ←
+607 GTGCACCTGG GGCTCOCOC TTCTCCOCCT TAGCACTGTG TCTCCGACA GCGGGCCACA
+667 CTGTTCTGCA CCOCTGGCAA TGCCGCAGCC TACCTTCGGT ATTTCTCACT AGAGGATGGC
+727 TCTGGCAGCC TTCTCCCTAT CATTAAACCCT TTTTCGCTCT TCTCCCTCCC TTCCCCTGTC
+787 AGCTCACCAA TGACTGTAAA TATATTTATC TATACTTATG GATACCCATT CACACCTTAT
+847 GCATCCAAAC ACTCCTGTCT CTTTCTCAGA CCAGACACCT TAGGCGTGGT TGACCTTAGG
+907 CGTGGTTGTC TTTTCTCCAG CATGTTGTTA GTGCTCCACA TGTC TCTGGA ACCATCTCTC
      Sp1
+967 TACTCTGGTG GCCTCTATAA TGGGGACTCT TCACCTCTTG GGGGTGTGCC TGGTTTTTGA
      →
+1027 A

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Competition assays showed that the bands were inhibited by both cold wild-type probe and cold mutated probe, although the inhibitory effect of the mutated probe was weaker (Figure 2c). These results suggest that the Int1(+12)A mutation reduces the gene's binding capacity for nuclear protein Sp1. To determine whether Sp1 is

involved in regulating the expression of the *F13A1* gene, the plasmid pN3-Sp1, expressing Sp1, was co-transfected. Compared with the transfection of pF13AInt+91, the co-transfection of pF13AInt+91 and pN3-Sp1 resulted in a 3.78-fold increase ( $P < 0.01$ ) in luciferase activity in U937 cells (Figure 2d). However, no difference in luciferase

**Figure 2** Sequence from position –114 of the promoter to position +951 of intron 1 of the *F13A1* gene, electrophoretic mobility shift assay analysis of the sequence at the exon 1/intron 1 border and Sp1 co-transfection analysis. (a) The nucleotide sequence of 114 bp of the minimum promoter, exon 1, and the first 951 nucleotides at the 5' end of the first intron. The transcription start site is indicated by an asterisk. Left numbering is relative to the transcription start site. The first exon/intron boundary is separated by a square bracket and the position of Int1(+12) is indicated by a vertical arrow. Five putative binding sites for Sp1 are shaded. The core sequence of the putative Sp1-binding site is underlined. The arrows indicate the directions of the Sp1 sites. (b) Gel-shift analysis of the sequence at the exon 1/intron 1 border covering the +12A mutation hybridized with the wild-type probe (WP30) or the mutant probe (MP30). The arrow indicates the observed gel shifts. (c) Gel-shift analysis of the sequence at the exon 1/intron 1 border in the absence or presence of a 125-fold excess of cold probe. (d) The relative luciferase activities of constructs pF13AInt1+91 and pF13AInt1+91mt12 in the absence or presence of a co-transfected construct expressing Sp1. \*\* Indicates a statistically significant difference ( $P < 0.01$ ) between the cells transfected with pF13AInt1+91 plus Sp1 and pF13AInt1+91. ## Indicates that the relative luciferase activity in cells transfected with pF13AInt1+91 plus Sp1 is significantly different from the relative luciferase activity of cells transfected with pF13AInt1+91mt12 or pF13AInt1+91mt12 plus Sp1 ( $P < 0.01$ ).

activity was observed between U937 cells transfected with pF13AInt+91mt12 plus pN3-Sp1 and those transfected with only pF13A+91mt12 (Figure 2d). Therefore, the Sp1-binding site between nucleotides +8 and +15 in intron 1 of the *F13A1* gene is involved in regulating the expression of FXIII. Therefore, the Int1(+12)A mutation causes the inefficient transcription of *F13A1* gene.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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- 1 Karimi, M., Berczyk, Z., Cohan, N. & Muszbek, L. Factor XIII deficiency. *Semin. Thromb. Hemost.* **35**, 426–438 (2009).
- 2 Hsieh, L. & Nugent, D. Factor XIII deficiency. *Haemophilia* **14**, 1190–1200 (2008).
- 3 Ichinose, A. Physiopathology and regulation of factor XIII. *Thromb. Haemost.* **86**, 57–65 (2001).
- 4 Anwar, R. & Miloszewski, K. J. A. Factor XIII deficiency. *Br. J. Haematol.* **107**, 468–484 (1999).

- 5 Trinh, C. H., Elsayed, W., Eshghi, P., Miri-Moghaddam, E., Zadeh-Vakili, A., Markham, A. F. *et al.* Molecular analysis of sixteen unrelated factor XIII deficient families from south-east of Iran. *Br. J. Haematol.* **140**, 578–592 (2008).
- 6 Castaman, G., Giacomelli, S. H., Ivaskevicius, V., Schroeder, V., Kohler, H. P., Dragani, A. *et al.* Molecular characterization of five Italian families with inherited severe factor XIII deficiency. *Haemophilia* **14**, 96–102 (2008).
- 7 Trigui, N., Frère, C., D'Ercole, C., Chambost, H., Chapuis, N., Pouymayou, C. *et al.* Molecular characterization of a novel mutation in the factor XIII A subunit gene associated with a severe defect and an adulthood diagnosis. *Haemophilia* **13**, 221–222 (2007).
- 8 Ariens, R. A. S., Kohler, H. P., Mansfield, M. W. & Grant, P. J. Subunit antigen and activity levels of blood coagulation factor XIII in healthy individual. Relation to sex, age, smoking and hypertension. *Arterioscler. Thromb. Vasc. Biol.* **19**, 2012–2016 (1999).
- 9 Kida, M., Souri, M., Yamamoto, M., Saito, H. & Ichinose, A. Transcriptional regulation of cell type-specific expression of the TATA-less A subunit gene for human coagulation factor XIII. *J. Biol. Chem.* **274**, 6138–6147 (1999).
- 10 Attié-Castro, F. A., Zago, M. A., Lavinha, J., Elion, J., Rodriguez-Delfin, L., Guerreiro, J. F. *et al.* Ethnic heterogeneity of the factor XIII Val34Leu polymorphism. *Thromb. Haemost.* **84**, 601–603 (2000).
- 11 Heng, C. K., Lal, S., Saha, N., Low, P. S. & Kamboh, M. I. The impact of factor XIIIa V34L polymorphism on plasma factor XIII activity in the Chinese and Asian Indians from Singapore. *Hum. Genet.* **114**, 186–191 (2004).
- 12 Rohrer, J. & Conley, M. E. Transcriptional regulatory elements within the first intron of Bruton's tyrosine kinase. *Blood* **91**, 214–221 (1998).
- 13 Shamsher, M. K., Chuzhanova, N. A., Friedman, B., Scopes, D. A., Alhaq, A., Millar, D. S. *et al.* Identification of an intronic regulatory element in the human protein C (PROC) gene. *Hum. Genet.* **107**, 458–465 (2000).
- 14 Warnecke, C., Willich, T., Holzmeister, J., Bottari, S. P., Fleck, E. & Regitz-Zagrosek, V. Efficient transcription of the human angiotensin II type 2 receptor gene requires intronic sequence elements. *Biochem. J.* **340**, 17–24 (1999).
- 15 Tanaka, S. & Ramirez, F. The first intron of the human  $\alpha 2(I)$  collagen gene (*COL1A2*) contains a novel interferon- $\gamma$  responsive element. *Matrix. Biol.* **26**, 185–189 (2007).
- 16 Girousse, A., Tavernier, G., Tiraby, C., Lichtenstein, L., Iacovoni, J. S., Mairal, A. *et al.* Transcription of the human uncoupling protein 3 gene is governed by a complex interplay between the promoter and intronic sequences. *Diabetologia* **52**, 1638–1646 (2009).

Supplementary Information accompanies the paper on Journal of Human Genetics website (<http://www.nature.com/jhg>)