

Molecular genetics of hereditary prothrombin deficiency in Indian patients: identification of a novel Ala362 → Thr (Prothrombin Vellore 1) mutation

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Summary. Prothrombin deficiency is a rare (1:200 000) autosomal recessive disorder caused by diverse mutations in prothrombin gene. We have studied the molecular basis of this disorder in four unrelated Indian patients. The diagnosis was based on prolonged prothrombin (PT) and activated partial thromboplastin times and low factor II coagulant activity (FII: C) measured using a PT based assay. FII: C levels ranged between 4.7% and 17.5%. Mutations were identified in all the four patients. Five different causative mutations including four (80%) missense and an in-frame deletion (20%) were identified. One of them was a novel, Ala362 → Thr aminoacid change affecting 'B' chain of α -thrombin. This mutation was present in a compound heterozygous state with a previously reported Arg-1 → Gln missense change affecting pro-peptide cleavage site. Ala362 → Thr occurred at a codon, evolutionarily conserved in all the 24 different prothrombins or its related serine proteases studied. Molecular modeling of this mutation was found to cause a conformational change around the region involving a catalytic triad residue His363 and a cysteine residue at codon 364. The FII: C level in this patient was 17.5%. Three other previously reported mutations were also detected in the homozygous state: Arg271 → Cys in Kringle-2 region, a Glu309 → Lys in 'A' chain of α -thrombin and an in-frame deletion of 3 bp (AAG) leading to Del Lys301/302 in 'A' chain of α -thrombin. This is the first report of the molecular basis of prothrombin deficiency in Indian patients and we suggest the eponym 'Prothrombin Vellore 1' for Ala362 → Thr mutation.

Keywords: conformation sensitive gel electrophoresis, India, prothrombin deficiency.

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Introduction

Prothrombin (factor II) is a vitamin-K dependent glycoprotein synthesized in liver and circulates in plasma at concentrations of 1–2 μ M. It is a precursor of the serine protease thrombin, a central enzyme in the process of hemostasis and thrombosis that exhibits both pro- and anticoagulant activities [1]. Prothrombin is activated by factor Xa in the presence of phospholipids, calcium and factor Va to form thrombin that converts fibrinogen to insoluble fibrin [1]. Thrombin promotes blood coagulation by stimulating platelet aggregation and activating factors V, VIII and XIII. It also inhibits coagulation by activating protein C and thrombomodulin. Thrombin has additional properties including cell proliferation [2,3] chemotaxis [4], tissue repair and angiogenesis [5,6].

Prothrombin is encoded by a gene which is 21 kb in length and located in chromosome 11p11-q12 [7,8]. It is composed of 14 exons separated by 13 introns [7]. This gene is transcribed into a prepropeptide containing protein of 622 aminoacids. Exons 1 and 2 encode the prepropeptide region (residues –43 to –1), exons 2 and 3 encode the γ -carboxyglutamic acid (Gla) domain (residues 1–40), exons 3–7 for kringle-1 domain (residues 41–155) and exons 7 and 8 for kringle-2 domain (residues 156–271). Exons 8–9 (residues 272–320) encode the light chain of thrombin and exons 9–14 encode the catalytic serine protease domain (residues 321–579). Prothrombin is activated by factor Xa by cleavage at Arg271-Thr272 and Arg320-Ile321 and by α -thrombin by cleavage at Arg284-Thr285. The resulting α -thrombin molecule is composed of an A-chain (Thr285-Arg320) bridged to a B-chain (Ile321-Glu579) by a disulfide bond formed by Cys293-Cys439. Mutations affecting most of these domains have been reported to cause prothrombin deficiency [9–11].

Congenital deficiency of prothrombin is an extremely rare autosomal recessive bleeding disorder affecting 1:2 000 000 in the general population [10]. Bleeding symptoms vary from severe spontaneous life or limb threatening hemorrhages or to mild presentations such as nose bleeding, menorrhagia and post-traumatic/surgical bleeding only [9,10]. Often there is a

discrepancy between the clinical severity of this disease and antigen levels or coagulant activity [9]. The molecular basis of this disease appears to be diverse in the few ($n = 34$) mutations characterized in prothrombin gene so far [11]. A vast majority of them are missense mutations (79%, $n = 27$) although insertion/deletions (12%, $n = 4/34$), nonsense (6%, $n = 2$) and splice site (3%, $n = 1$) mutations have also been described [11]. Identification of additional mutations in this gene is important, as they constitute naturally occurring models for studying the molecular basis of this disease and to understand the effect of these mutations on the function of thrombin, a key enzyme in blood coagulation. Moreover, such data can also help in precise genetic diagnosis and genetic counseling in families affected by this disorder. Currently, most of the mutational data available in the literature has come from patients of Caucasian [9,12–18], Middle Eastern [9] or Oriental [19,20] origin. In populations (South India) where consanguineous marriages are often customary, the frequency of such recessively inherited coagulation disorders in homozygosity is significantly (eight- to 10-fold) increased [21]. We report here for the first time, the clinical, phenotypic and molecular data in Indian patients with congenital prothrombin deficiency.

Patients and methods

Patients

Genomic DNA from four unrelated South-Indian patients was studied after informed consent. Their clinical severity was graded as described [9]. Severe bleeders were those with a history of spontaneous life or limb threatening bleedings such as hemarthroses, hematomas, GI and CNS bleedings. Mild bleeders were those who bled only after trauma or surgery or had epistaxis, easy bruising or menorrhagia.

Factor II assays

Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured on a CA1500 automated analyzer (Sysmex, Hyogo, Japan). FII coagulant (FII: C) activity was determined by a prothrombin time based one-stage assay using FII deficient plasma and recombinant human thromboplastin (Innovin[®], Dade Behring, Marburg, Germany). FII antigen levels were not determined in any of these patients, because of the non-availability of appropriate samples at the time of this study.

Prothrombin gene amplification

Prothrombin gene exonic and their flanking intronic regions and 3' untranslated (UTR) region was amplified by 12 pairs of primers (Table 1) designed by Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Polymerase chain reaction (PCR) was performed in a 25 μ L reaction volume containing 7.5 pmoles of each primer in a 1X concentration of a ready reaction mix (ABgene[®], Epsom, UK) containing 1.5 mM MgCl₂, 75 mM Tris HCl (pH 8.8), 20 mM

(NH₂)₂SO₄, 0.2 mM of each of dNTP, 0.01% (v/v) Tween 20 and 1.25 units of Thermoprime plus DNA polymerase. Approximately 250 ng of genomic DNA was used for amplification reactions. Following an initial denaturation at 94 °C for 5 min, 30 cycles of PCR amplification was performed, with denaturation at 94 °C for 40 s, annealing at 58 °C for 40 s and extension at 72 °C for 40 s. The final extension was at 72 °C for 5 min.

Conformation sensitive gel electrophoresis

Heteroduplexes were prepared by mixing 5 μ L of PCR product from each patient with 5 μ L of PCR product from a normal control. Samples were denatured by heating to 95 °C for 8 min and then incubated at 50 °C for 45 min to allow heteroduplex formation [22]. Three to five microliters of heteroduplexed samples were mixed with 1.5 μ L of loading buffer (70% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue, 0.01% 1 M EDTA) and electrophoresed in a mildly denaturing gel (400 × 330 × 1 mm in size) containing 10% acrylamide [99.1 acrylamide (Invitrogen Lifetechnologies, Groningen, The Netherlands); bisacryloylpiperazine (Fluka Chemie, Buchs, Switzerland)], 10% ethylene glycol (Sigma, St Louis, MO, USA), 15% formamide (Sigma) and 0.5X TTE buffer [20X TTE -1.78 M Tris (USB, Cleveland, OH, USA), 570 mM Taurine (USB), 4 mM EDTA (USB)] as described previously [22]. Polymerization was achieved by the addition of 0.1% ammonium persulphate (USB), and 0.07% N, N, N', N'-tetramethylethylenediamine (TEMED) (Sigma). Following electrophoresis at 400 V for 18 h, bands were visualized by ethidium bromide staining.

DNA sequencing

Samples displaying abnormal conformation sensitive gel electrophoresis (CSGE) patterns were sequenced by the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) on an ABI 310 genetic analyzer (PE Applied Biosystems, Foster City, CA, USA) using the same primers described for PCR (Table 1). Mutation nomenclature in prothrombin gene was used as previously described [7].

Structural analysis of a novel missense mutation

A novel missense mutation was studied based on the coordinates (identifier 1 ppb) for the human α -thrombin or on the representational structure of human prothrombin [9] created using crystal structures of human α -thrombin (PDB: 1 ppb), bovine prothrombin fragment 1 (PDB: 2 hpq), human prothrombin fragment 2 (PDB: 2pf2) deposited with the protein data bank. The potential effects of this aminoacid substitution was modeled by SwissPdb Viewer software [23]. The final images were created by ViewerPro 4.2 software (Accelrys, <http://www.accelrys.com>).

The aminoacid sequences of prothrombins from 17 different species and seven related proteases were obtained from

Table 1 Primers used for amplification and sequencing of prothrombin gene

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
F2-EXON 1	ACCCTCTCCGCTGATTTCTT	GCCCTGAGCTCCTGACTTCT	323
F2-EXON 2 F	AGGGTGGGCTTGCTTCAT	CTCGCTTGCTTCTCTGTGG	326
F2-EXON 3 + 4 F	AAACAACACAAAACAGGAGCTG	CAAGCTCCCCTCCATGTC	420
F2-EXON 5 F	CAACTTTGCAGGGAGAGAGG	GCGGCAGAAAATTCTCCTGTA	321
F2-EXON 6 F	GAGAACAGGGAGCAAGCGTA	TGTGCCTGGGTAGCCAGTAT	300
F2-EXON 7 F	GTCTGTTCCGGTCCATGTGT	AGGATTTGTCCCTGCAACTC	421
F2-EXON 8 + 9 F	CTAGGGGATGGGTGAGGAAT	CCCCAGGAGTGAATGGTAG	439
F2-EXON 10 F	GGTTCTTAGACCTGGGATTG	GATCGCTTTGGAGGACTCAG	317
F2-EXON 11F	CTGTCTCCAGAACCCCAAG	AGAGCCAGGCCCAGAACT	322
F2-EXON 12F	CCAGCTCTGGCGTTTTAGAT	CTTGAACCCAGGCACAACCTC	358
F2-EXON 13F	TGTGTAGGGTGAGGAAGTGG	GTATTCCTTGCCAGGCAAC	300
F2-EXON 14 + 3UTR F	TGATGTGACCTTGAACCTTGACTC	CAGAGAGCTGCCCATGAATAG	332

SwissProt and Trembl databases (<http://us.expasy.org/sprot/>) using PSI-BLAST to study the conservation of an amino acid mutated by missense change. Their accession numbers are as follows: P00734 – human prothrombin precursor; P08709 – human coagulation factor VII precursor; P00740 – human coagulation factor IX precursor; P00742 – human coagulation factor X precursor; Q15146 – human plasminogen precursor; P04070 – human protein C precursor; P22891 – human protein Z precursor; P08519 – human apolipoprotein (a) precursor; Q7SXH8 – zebra fish hypothetical protein; Q91218 – thrombin, *Oncorhynchus mykiss*; Q90244 – thrombin, *Acipenser transmontanus*; Q804W7 – prothrombin precursor, *Fugu rubripes*; P00735 – bovine prothrombin precursor; AAR99595 – coagulation factor II, *Sus scrofa*; P19221 – mouse prothrombin precursor; P18292 – rat prothrombin precursor; Q91001 – chicken thrombin; Q9PTW7 – ostrich prothrombin; Q90WP0 – thrombin, *Trachemys scripta elegans*; Q90WT4 – African crocodile putative thrombin; Q90WS2 – putative thrombin, *Elaphe* sp.; Q91004 – thrombin, *Gecko gecko*; Q90387 – thrombin, *Cynops pyrrhogaster*; Q90504 – Pacific hagfish thrombin. Multiple sequence alignment was performed with CLUSTALW (<http://www.ebi.ac.uk/clustalw/>).

Results

Patients

The clinical features and hematological parameters of the patients are described in Table 2. Bleeding symptoms included easy bruising, post-traumatic bleeds, hemarthroses (50% each), epistaxis, gum bleeding, menorrhagia, dental extraction bleeding, hematemesis and hematomas (25% each). None of them had spontaneous severe bleeding. BL-90 and BL-156 had post-traumatic bleeding only. The clinical presentation in two patients did not correlate with FII: C. Severe bleeding symptoms were seen in-patient BL-154 with a coagulant activity of 10.2%. She had pubertal menorrhagia and had required multiple units of fresh blood during her first two childbirths. She had aborted during her third pregnancy following which the bleeding was unabated. It had required an emergency total abdominal hysterectomy with transfusion

support. She had fractured her right femur following a fall 4 years ago. A open reduction and internal fixation was planned elsewhere but could not be carried out because of severe bleeding which had been managed by fresh blood and fresh frozen plasma transfusion. The swelling in the right leg had increased progressively and she presented to us with a pseudo-tumor in right lower limb (Fig. 1). On evaluation she was found to have prothrombin deficiency. Patient BL-156 had a mild clinical phenotype with a reduced FII: C of 7.8%.

Mutation analysis

The PCR products from the patients mixed with normal controls were indistinguishable on agarose gels (data not shown). This suggested that only single base changes or micro-deletions/insertions were likely. CSGE based mutation screening and DNA sequencing identified four of five disease causing mutations and 10 different polymorphisms (Table 2). A G → A transition in the heterozygous condition at nucleotide 8825 and responsible for Ala362 → Thr mutation was not identified by CSGE. This mutation was detected by complete gene sequencing. The low sensitivity of CSGE to detect such G → A (C → T on reverse strand) transitions has been reported [24]. Of the 10 different polymorphisms identified, two (4282A → G, 4304insG) were novel. The remaining 8 (459delT, 554A → G, 4048T → C, 4125C → G, 4203C → T, 4272G → A, 4291A → G, 4298A → G) polymorphisms have been essentially described among Japanese [25,26] or Italian [12] patients. Most (6 of 8) of them occurred in a region containing exon 6 and introns E and F (nucleotides 4048–4303) that has been known to have high sequence variability [25]. Apart from these nucleotide variations, five different disease-causing mutations were also found in four unrelated patients (Fig. 2). Four (80%) of them were missense and one (20%) was an in-frame deletion. One of them was a novel mutation.

Novel mutation

A novel G → A transition at nucleotide position 8825 in exon 10 in a heterozygous state was identified in one of the patients

Table 2 Clinical features and mutation data on patients with inherited prothrombin deficiency

UPN	AOI/sex	Con [†]	Family history	PT (s) control (10.5–11.2)	APTT (s) Control (28.2–30.7)	FII:C	Bleeding symptoms	Domain	Exon/intron	Nucleotide	Mutation/codon*	Aminoacid	Restriction site altered	Comments
BL-75	32/Male	Yes	Sporadic	25.8	53.7	4.7%	Severe	α -thrombin	9 Intron E	7484/7489 4048	Del GAA T → C	Del Lys301/302	NA	Homozygous
BL-90	4/Male	No	Sporadic	14.4	36.9	17.5%	Mild	Pro-peptide α -thrombin	2 10 Intron A	514 8825 459	CGA → CAA GCC → ACC [†] Del T	Arg-1 → Gln Ala362 → Thr	NA Taul-	Homozygous Heterozygous Heterozygous
BL-154	60/Female	No	Sporadic	19.8	65.4	10.2%	Severe	Pro-peptide Kringle-2	2 Intron E Intron F Intron F Intron F Intron F Intron F	554 4048 4272 4282 4291 4298 4304	CTA → CTG T → C G → A A → G [†] A → G A → G Ins G [†]	Leu13 → Leu	BstNI +	Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous
BL-156	2/Male	Yes	Familial	25.9	72.4	7.8%	Mild	α -thrombin	8 Intron E Intron E 6 Intron F Intron F Intron F Intron F	7311 4048 4125 4203 4272 4291 4298 4304 7509 4048	CGT → TGT T → C C → G ACG → ATG G → A A → G A → G Ins G [†] GAA → AAA T → C	Arg271 → Cys Thr122 → Met	SpII- NcoI +	Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous

AOI, age at first investigation; Con, consanguinity status; PT, prothrombin time; APTT, activated partial thromboplastin time; FII:C, Factor II coagulant (FII: C) activity; +, mutation creates a restriction site; -, mutation abolishes a restriction site for the restriction enzyme; NA, restriction enzyme not available.

*Nucleotide numbering according to Degen and Davie [7].

[†]Novel mutations/polymorphisms, not reported previously in Peyvandi *et al.* [10] or in <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Details&DB=PubMed>.

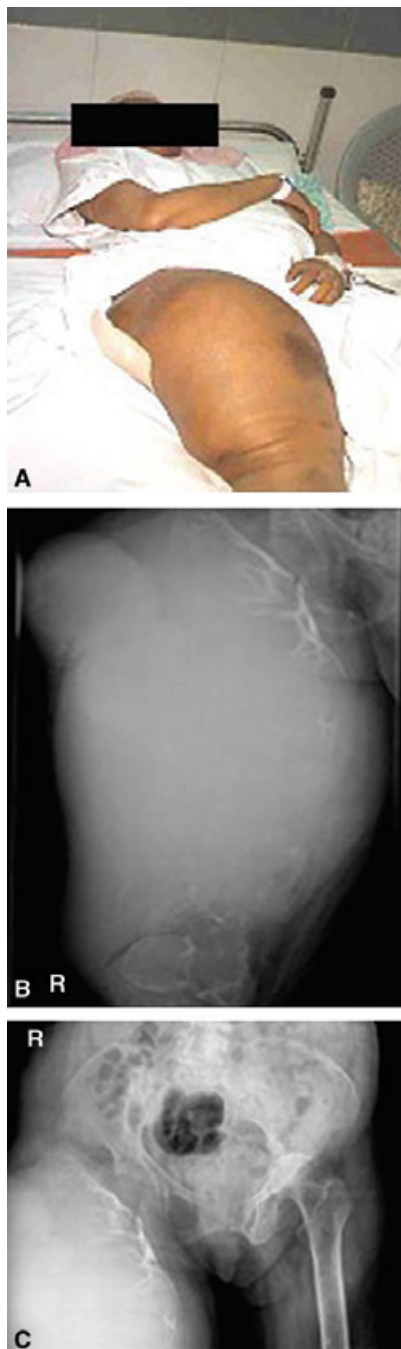


Fig. 1. (A) The clinical photograph of pseudo-tumor of right thigh in patient BL-154; (B) the radiological picture of destruction of right femur by this pseudo-tumor; (C) the destruction of right-sided pelvic joint in this patient.

(BL-90). This predicts the replacement of alanine-362 by threonine in B-chain of α -thrombin.

Reported mutations

In the pro-peptide sequence in exon 2 of prothrombin gene, a 514G \rightarrow A transition was responsible for an Arg-1 \rightarrow Gln missense change. This mutation had occurred in a compound

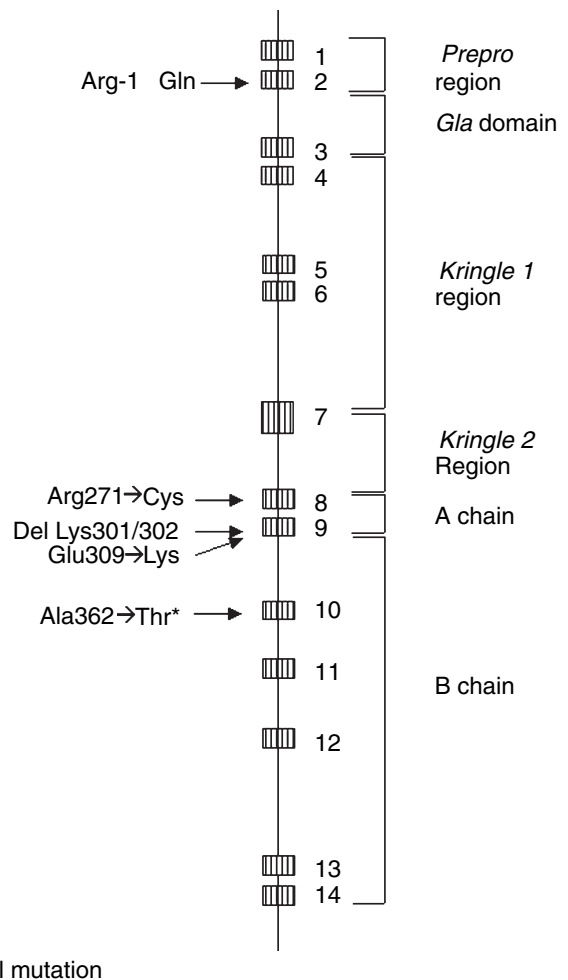


Fig. 2. Schematic representation of prothrombin gene mutations identified in this study.

heterozygous state with an Ala362 \rightarrow Thr mutation in patient BL-90. An Arg271 \rightarrow Cys aminoacid change detected in patient BL-154 corresponds to Kringle-2 region. This substitution arises because of a 7311C \rightarrow T transition in a homozygous form in exon 8. The remaining two reported mutations were present in a homozygous condition in exon 9 and affected the A chain of α -thrombin (Fig. 3). An in frame-deletion of 3 bp (7484/7489 del GAA) leading to deletion of one of either Lys301 or Lys302 was present in patient BL-75. The second mutation in this region was a Glu309 \rightarrow Lys (Prothrombin 'Denver') aminoacid change caused by a 7509G \rightarrow A transition (BL-156).

Discussion

Congenital abnormalities of prothrombin are rare. Only 34 different mutations in this gene have been characterized so far [11]. This is also the first study describing mutations in this gene from India. Only two other large studies – one including Italian, Iranian [9] and the other with patients from Puerto Rico [13] population describing patients with prothrombin deficiency have been published. Our strategy for the identifi-

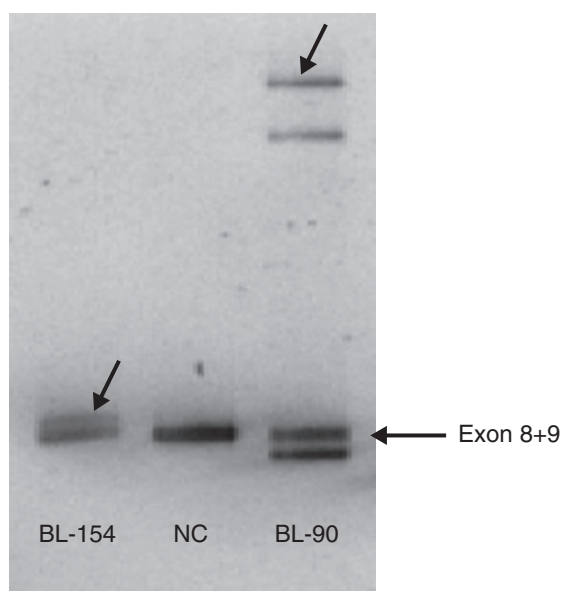


Fig. 3. Representative gel photograph of conformation sensitive gel electrophoresis (CSGE) analysis for polymerase chain reaction (PCR) (exonic) fragment 8 + 9 of prothrombin gene: Patient PCR products were mixed with equal volume of normal control (NC) PCR product and analyzed in a 10% CSGE gel. The abnormal mobility of patient PCR product relative to a normal control is shown by an arrow. Shown here are a G → A transition at nucleotide 7509 (Glu309 → Lys) in patient BL-154 and 7484/7489 del GAA (Del Lys 301/302) mutation in patient BL-90.

cation of causal mutations was to screen for mutations by a new CSGE based method followed by DNA sequencing. The exonic and their flanking intronic regions and 3'UTR regions in the prothrombin genes of the probands were screened, as these are most likely sites for mutations that would affect transcription or translation. Causative mutations were identified in each of these patients. All but one patient had homozygous mutations permitting the identification of the clinical consequence of such mutations.

Of the five different causative mutations identified (Fig. 2), four were missense and the other an in-frame deletion (7484/7489 del GAA) in exon 9. None of these mutations are predicted to result in a truncated protein or no protein at all. This is in agreement with previous mutation data [9] and consistent with the hypothesis that a complete absence of prothrombin is incompatible with life as has been demonstrated in a knockout mice model [27]. A majority of mutations detected in this series were present in catalytic domain (60%) emphasizing the functional importance of this region. Mutations were also located in 'Pro-peptide' (20%) and 'Kringle-2' (33%) domains suggesting that all these regions are important in maintaining the overall structure and function of prothrombin. One of these mutations identified in the catalytic domain was novel.

The novel 8825G → A mutation was responsible for an alanine to threonine change at codon 362. As this is the first report of the molecular basis of prothrombin deficiency in Indian patients, we propose the eponym 'Prothrombin Vellore 1' for Ala362 → Thr mutation. This mutation did not result in

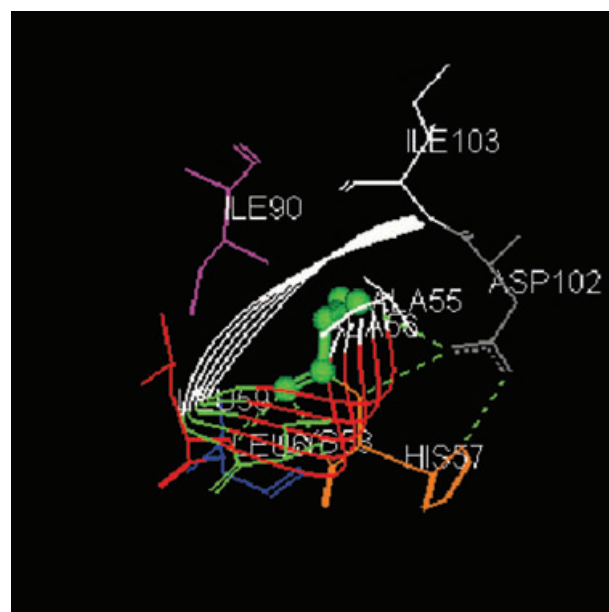


Fig. 4. Close-up of a region of Ala362 [PDB: 1 ppb, Chymotrypsinogen numbering (CT): 56] in human α -thrombin. Ala362 (shown in green) is a hydrophobic aminoacid located buried (Solvent accessibility-0 [9]) in the hydrophobic stretch between aminoacids Trp357 (CT: 51) to Leu365 (CT: 59) involving β -strand D in the serine protease domain. Alanine at codon 362 is spatially close to the catalytic triad residue His363 (CT: 57) and is hydrogen bonded to Ala361 (CT: 55), Cys364 (CT: 58), Leu365 (CT: 59), Leu366 (CT: 60), Ile406 (CT: 90), Asp419 (CT: 102) and Ile420 (CT: 103). This hydrophobic environment is further enhanced by the presence of a buried disulfide bridge between adjacent residue Cys364 and Cys348 (CT: 42). The replacement of Ala362 by threonine is likely to cause structural changes in this region. The side chain of threonine is much larger than alanine. As a result a conformational change in this region is likely between Trp357-Leu365 which contains His363 that is a part of the active site catalytic triad (Asp419 and Ser525) and also contains cysteine 364 which is disulfide bonded to cysteine at 348.

alternate splicing as predicted by the splice site prediction program (http://www.fruitfly.org/seq_tools/splice.html). Ala362 → Thr mutation is located within the histidine disulfide loop (residues 348–364) of the thrombin B chain (residues 321–579), that contains important structural features like the active site His363, the carbohydrate attachment site at Asn373 and the loop B sequence (residues 367–375) [9]. The histidine disulfide loop is known to be a conserved region in the serine protease domains of thrombin and homologous proteins [9]. Alignment of aminoacid sequences from 24 homologous prothrombins or related serine proteases shows that Ala362 is conserved in all of them. This indicates its importance to the structure of thrombin. The possible effect of this mutation on the crystal structure of human α -thrombin is detailed in Fig. 4. Further more, Ala362 → Thr was found to introduce additional hydrogen bonds with Ile406 and Tyr410 in human prothrombin. These structural perturbations in the mutant probably interfere with the autocatalytic cleavage of α -thrombin (Arg284-Thr285; Arg155-Ser156) or with the FXa activation (Arg320-Ile321) of prothrombin to thrombin as has been recently suggested for a Gly330Ser mutation [28]. However

further studies are needed to demonstrate the functional effect of this mutation. The reported identification of a topologically equivalent Ala220 → Val mutation in factor IX gene to cause hemophilia B [29], also provides evidence that mutations in this codon are pathognomic.

An Arg-1 → Gln change was detected in a compound heterozygous state with the novel Ala362 → Thr mutation. Arg-1 → Gln change has been reported [9] in a homozygous condition in an Iranian patient presenting with severe bleeding symptoms. This substitution involves the replacement of a highly basic residue by an uncharged hydrophilic amino acid [9]. It affects a highly conserved recognition sequence (-2Arg-Arg-Ala-Asn+2) for a propeptidase, furin. This recognition motif is present in all the six vitamin K dependent clotting factors namely prothrombin, factors VII, IX, X, protein C and S. A mutation at this site has been therefore postulated to abolish the cleavage of propeptide from mature prothrombin during its biosynthesis [9].

An Arg271 → Cys mutation was associated with a FII: C of 10.2% and severe bleeding symptoms in one of our patients. This mutation has been demonstrated in a Japanese patient with a similar severe phenotype and a corresponding coagulant activity of 18% (Prothrombin Obihiro) [25]. The prothrombin in this case has been reported to be dysfunctional, as the Arg271 → Cys mutation is located at a factor Xa cleavage site (Arg271-Thr272). Prothrombins Madrid [30] and Barcelona [31] also have identical Arg271 → Cys mutation, while prothrombins Padua [32] and Dhahran [33] have an Arg271 → His substitution. All these mutations are known to affect the activation of the prothrombin by factor Xa and subsequent generation of thrombin to cause excessive bleeding in these patients. Although a majority of the polymorphisms (Table 2) identified in our patient (BL-154) were reported in the Japanese patient, this mutation is likely to have an independent origin for two reasons. A novel 4304insG polymorphism was identified only in our patient and secondly, this mutation had occurred at a 'CpG' dinucleotide which is known hotspots for spontaneous demethylation of cytosine to thymidine nucleotide [25].

The in-frame deletion of one of the lysine residues at codon 301 or 302 found in this series has been reported among two Iranian kindred [9]. All these patients are characterized by severe bleeding symptoms. This mutation has been recently shown by expression studies to have a 20-fold decreased interaction with anti-thrombin, significantly reduced sensitivity to sodium ion, defective platelet activating receptor 1 activation, decreased interaction with protein C. Molecular dynamics simulation of the mutant thrombin in full explicit water solvent provided support to the role of the mutant A-chain in affecting conformation and catalytic properties of the B-chain, as well as in the geometry of the catalytic triad residues [34]. This in-frame deletion is consistent with a status of hypoprothrombinemia (FII: C of 1% and 7% observed in Iranians and 4.7% in Indian patient).

The substitution of glutamic acid at codon 309 by lysine corresponded to mild bleeding symptoms. Glu309 → Lys

(Prothrombin Denver II) has been shown to be associated with severe bleeding in a patient of Caucasian origin [35] when present in a compound heterozygous state with Glu300 → Lys substitution (Prothrombin Denver I). Glu309 residue has been described [35] as part of an A1 amphiphilic α -helix in the serine protease, with these hydrophobic residues interacting with the thrombin heavy chain. A change of negatively charged Glu309 for a positively charged Lys is believed to disrupt this structure and affect normal folding of the molecule. It has been hypothesized that this mutation could interfere with the autocatalytic cleavage of the A-chain of thrombin or factor Xa activation [35].

In conclusion, this report describes the mutations in four unrelated patients with inherited prothrombin deficiency adding significantly to the data on the molecular basis of this condition.

References

- 1 Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* 1991; **30**: 10363–70.
- 2 Bar-Shavit R, Kahn AJ, Mann KG, Wilner GD. Identification of a thrombin sequence with growth factor activity on macrophages. *Proc Natl Acad Sci USA* 1986; **83**: 976–80.
- 3 Zetter BR, Antoniades HN. Stimulation of human vascular endothelial cell growth by a platelet-derived growth factor and thrombin. *J Supramol Struct* 1979; **11**: 361–70.
- 4 Seino Y, Ikeda U, Ikeda M, Yamamoto K, Misawa Y, Hasegawa T, Kano S, Shimada K. Interleukin 6 gene transcripts are expressed in human atherosclerotic lesions. *Cytokine* 1994; **6**: 87–91.
- 5 Fenton JW II. Regulation of thrombin generation and functions. *Semin Thromb Haemost* 1988; **14**: 234.
- 6 Tsopanoglou NE, Maragoudakis ME. On the mechanism of thrombin-induced angiogenesis. Potentiation of vascular endothelial growth factor activity on endothelial cells by up-regulation of its receptors. *J Biol Chem* 1999; **274**: 23969–76.
- 7 Degen SJ, Davie EW. Nucleotide sequence of the gene for human prothrombin. *Biochemistry* 1987; **26**: 6165–77.
- 8 Royle NJ, Irwin DM, Koschinsky ML, MacGillivray RT, Hamerton JL. Human genes encoding prothrombin and ceruloplasmin map to 11p11-q12 and 3q21-24, respectively. *Somat Cell Mol Genet* 1987; **13**: 285–92.
- 9 Akhavan S, Mannucci PM, Lak M, Mancuso G, Mazzucconi MG, Rocino A, Jenkins PV, Perkins SJ. Identification and three-dimensional structural analysis of nine novel mutations in patients with prothrombin deficiency. *Thromb Haemost* 2000; **84**: 989–97.
- 10 Peyvandi F, Duga S, Akhavan S, Mannucci PM. Rare coagulation deficiencies. *Haemophilia* 2002; **8**: 308–21.
- 11 Mannucci PM, Duga S, Peyvandi F. Recessively inherited coagulation disorders. *Blood* 2004; **104**: 1243–52.
- 12 Poort SR, Landolfi R, Bertina RM. Compound heterozygosity for two novel missense mutations in the prothrombin gene in a patient with a severe bleeding tendency. *Thromb Haemost* 1997; **77**: 610–5.
- 13 Lefkowitz JB, Weller A, Nuss R, Santiago-Borrero PJ, Brown DL, Ortiz IR. A common mutation, Arg457 → Gln, links prothrombin deficiencies in the Puerto Rican population. *J Thromb Haemost* 2003; **1**: 2381–8.
- 14 Akhavan S, Luciani M, Lavoretano S, Mannucci PM. Phenotypic and genetic analysis of a compound heterozygote for dys- and hypoprothrombinemia. *Br J Haematol* 2003; **120**: 142–4.
- 15 Sun WY, Burkart MC, Holahan JR, Degen SJ. Prothrombin San Antonio: a single amino acid substitution at a factor Xa activation site (Arg320 to His) results in dysprothrombinemia. *Blood* 2000; **95**: 711–4.

- 16 Sun WY, Ruiz-Saez A, Burkart MC, Bosch N, Degen SJ. Prothrombin carora: hypoprothrombinaemia caused by substitution of Tyr-44 by Cys. *Br J Haematol* 1999; **105**: 670–2.
- 17 Poort SR, Njo KT, Vos HL, Bertina RM. Two novel mutations in the prothrombin gene cause severe bleeding in a compound heterozygous patient. *Blood Coagul Fibrinolysis* 1998; **9**: 761–4.
- 18 Henriksen RA, Dunham CK, Miller LD, Casey JT, Menke JB, Knupp CL, Usala SJ. Prothrombin Greenville, Arg517 → Gln, identified in an individual heterozygous for dysprothrombinemia. *Blood* 1998; **91**: 2026–31.
- 19 Wang W, Fu Q, Zhou R, Wu W, Ding Q, Hu Y, Wang X, Wang H, Wang Z. Prothrombin Shanghai: hypoprothrombinaemia caused by substitution of Glu29 by Gly. *Haemophilia* 2004; **10**: 94–7.
- 20 Iwahana H, Yoshimoto K, Shigekiyo T, Shirakami A, Saito S, Itakura M. Molecular and genetic analysis of a compound heterozygote for dysprothrombinemia of prothrombin Tokushima and hypoprothrombinemia. *Am J Hum Genet* 1992; **51**: 1386–95.
- 21 Peyvandi F. *Rare Coagulation Disorders*, PhD Thesis. Milan: University of Milan, 2000.
- 22 Williams IJ, Abuzenadah A, Winship PR, Preston FE, Dolan G, Wright J, Peake IR, Goodere AC. Precise carrier and prenatal diagnosis in families with haemophilia A: use of conformation sensitive gel electrophoresis for rapid mutation screening and polymorphism analysis. *Thromb Haemost* 1998; **79**: 723–6.
- 23 Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997; **18**: 2714–23.
- 24 Eng C, Brody LC, Wagner TM, Devilee P, Vijg J, Szabo C, Tavtigian SV, Nathanson KL, Ostrander E, Frank TS. Interpreting epidemiological research: blinded comparison of methods used to estimate the prevalence of inherited mutations in BRCA1. *J Med Genet* 2001; **38**: 824–33.
- 25 Miyata T, Zheng YZ, Kato A, Kato H. A point mutation (Arg271 → Cys) of a homozygote for dysfunctional prothrombin, prothrombin Obihiro, which has a region of high sequence variability. *Br J Haematol* 1995; **90**: 688–92.
- 26 Iwahana H, Yoshimoto K, Itakura M. NcoI RFLP in the human prothrombin (F2) gene. *Nucleic Acids Res* 1991; **19**: 4309.
- 27 Sun WY, Witte DP, Degen JL, Colbert MC, Burkart MC, Holmback K, Xiao Q, Bugge TH, Degen SJ. Prothrombin deficiency results in embryonic and neonatal lethality in mice. *Proc Natl Acad Sci USA* 1998; **95**: 7597–602.
- 28 Akhavan S, Miteva MA, Villoutreix BO, Venisse L, Peyvandi F, Mannucci PM, Guillin MC, Bezeaud A. A critical role for Gly25 in the B chain of human thrombin. *J Thromb Haemost* 2005; **3**: 139–45.
- 29 Hemophilia B Mutation Database-version 13. 2004. A database of point mutations and short additions and deletions in the factor IX gene. <http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html/>.
- 30 Diuguid DL, Rabiet MJ, Furie BC, Furie B. Molecular defects of factor IX Chicago-2 (Arg 145 – His) and prothrombin Madrid (Arg 271 – cys): arginine mutations that preclude zymogen activation. *Blood* 1989; **74**: 193–200.
- 31 Rabiet MJ, Furie BC, Furie B. Molecular defect of prothrombin Barcelona. Substitution of cysteine for arginine at residue 273. *J Biol Chem* 1986; **261**: 15045–8.
- 32 James HL, Kim DJ, Zheng DQ, Girolami A. Prothrombin Padua I: incomplete activation due to an amino acid substitution at a factor Xa cleavage site. *Blood Coagul Fibrinolysis* 1994; **5**: 841–4.
- 33 O'Marcaigh AS, Nichols WL, Hassinger NL, Mullins JD, Mallouh AA, Gilchrist GS, Owen WG. Genetic analysis and functional characterization of prothrombins Corpus Christi (Arg382-Cys), Dhahran (Arg271-His), and hypoprothrombinemia. *Blood* 1996; **88**: 2611–8.
- 34 De Cristofaro R, Akhavan S, Altomare C, Carotti A, Peyvandi F, Mannucci PM. A natural prothrombin mutant reveals an unexpected influence of A-chain structure on the activity of human alpha-thrombin. *J Biol Chem* 2004; **279**: 13035–43.
- 35 Lefkowitz JB, Haver T, Clarke S, Jacobson L, Weller A, Nuss R, Manco-Johnson M, Hathaway WE. The prothrombin Denver patient has two different prothrombin point mutations resulting in Glu-300 → Lys and Glu-309 → Lys substitutions. *Br J Haematol* 2000; **108**: 182–7.