bortezomib and dexamethasone are warranted.[2,3] The most common toxicities were gastrointestinal symptoms, thrombocytopenia, neutropenia, fatigue and peripheral neuropathy (Figure 2). The majority of the side effects were low grade. The thrombocytopenia was transient, with recovery within the 10-day rest period in each cycle, and was not associated with bleeding complications. Six patients had herpes zoster. The dose of bortezomib was reduced in 18 patients and the drug was discontinued in 12 patients because of side effects: peripheral neuropathy (n=6), gastrointestinal symptoms (n=4), skin rash (n=1) and orthostatic hypotension (n=1).

In our observational analysis, the response rate to bortezomib was comparable to those in two prospective clinical studies, CREST and SUMMIT, which reported response rates of 35-50%.[4,5] The median duration of response was 9 months. In the CREST study, the median duration of response was 9.5 months and 13.7 months in the 1.0 mg/m$^2$ and 1.3 mg/m$^2$ groups, respectively, while in the SUMMIT study the median response duration was 12 months. A possible explanation for the shorter duration of response in our series may be that dose reduction was performed more frequently by local physicians in our study than in CREST and SUMMIT, which were conducted in expert myeloma centers. Therefore, the clinical practice in community hospitals of reducing doses in patients with treatment-related toxicity without adherence to well-defined guidelines may jeopardize the outcome of the efficacy of bortezomib in multiple myeloma.

A remarkable observation in our series was the high incidence of herpes zoster. Six patients developed a herpes zoster infection during treatment with bortezomib. The transcription factor NF-κB has been demonstrated to play a pivotal role in cytokine signaling and the generation of cell-mediated immune response in numerous models.[6,7] Therefore, inhibition of NF-κB may increase the risk of reactivation of the varicella zoster virus. Prophylactic antiviral medication should be considered in predisposed patients who receive bortezomib.

In conclusion, bortezomib can induce marked and durable response in advanced multiple myeloma. Overall, bortezomib was well tolerated and the toxicity was acceptable.

*Ka Lung Wu,* Wessel van Wieringen,° Edo Vellenga,# Sonja Zweegman,@ Henk M. Lokhorst,^ Pieter Sonneveld**

**Erasmus MC, Department of Hematology, Rotterdam, The Netherlands; °Erasmus MC, HOVON Data Center, Rotterdam, The Netherlands; #University Medical Center, Department of Hematology, Groningen, The Netherlands; @VU Medical Center, Department of Hematology, Amsterdam, the Netherlands; ^University Medical Center, Department of Hematology, Utrecht, The Netherlands*

*Participating hospitals in this study: Meander Medical Center, Amersfoort, MHH Kramer; VU Medical Center, Amsterdam, S Zweegman; Medical Spectrum Twente, Enschede, WM Smit; University Medical Center Groningen, E Vellenga; Leiden University Medical Center, RM Barge; Academic Hospital Maastricht, Maastricht, GM Bos; University Medical Center Nijmegen, RAP Raymakers; University Medical Center Utrecht, HM Lokhorst; Erasmus Medical Center, Rotterdam, KL Wu, P Sonneveld.*

*Key words: bortezomib, multiple myeloma, toxicity, herpes zoster.*

*Correspondence: Peter Sonneveld, Erasmus MC, Department of Hematology, Room L-407, PO Box 2040, 3000 CA Rotterdam. Phone: international +31.10.4633312. Fax: international +31.10.4635814. E-mail: p.sonneveld@erasmusmc.nl*

## References

1. Blade J, Samson D, Reece D, Apperley J, Bjorkstrand B, Gahrton G, et al. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. Br J Haematol 1998;102:1115-23.
2. Mitsiades N, Mitsiades CS, Richardson PG, Poulaki V, Tai YT, Chauhan D, et al. The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications. Blood 2003;101:2377-80.
3. Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res 2001;61:3071-6.
4. Jagannath S, Barlogie B, Berenson J, Siegel D, Irwin D, Richardson PG, et al. A phase 2 study of two doses of bortezomib in relapsed or refractory myeloma. Br J Haematol 2004; 127:165-72.
5. Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 2003;348:2609-17.
6. Barnes PJ, Karin M. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997; 336:1066-71.
7. Wulczyn FG, Krappmann D, Scheidereit C. The NF-κ B/Rel and IκB gene families: mediators of immune response and inflammation. J Mol Med 1996;74:749-69.



*Disorders of Hemostasis*

## Exon skipping partially restores factor VIII coagulant activity in patients with mild hemophilia A with exon 13 duplication

**Ectopic mRNA was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) in patients with duplication of *F8* gene exon 13, a mutation which has been demonstrated to be a cause of mild hemophilia A in 32% of Northern Italian subjects. Two different transcripts originate from mutated genomic DNA, due to alternative splice processes. The larger-sized transcript contains both duplicated exons 13, the smaller one contains only one exon 13. The residual FVIII:C activity which accounts for the mild hemophilia A phenotype derives from the latter transcript.**

haematologica 2005; 90:997-999
(http://www.haematologica.org/journal/2005/7/997.html)

We described the duplication of exon 13 as a disease causative mutation in a cohort of Northern Italian patients with hemophilia A (HA).[1] The association of the alteration with a residual FVIII coagulant activity >8% suggested that a certain amount of normal protein was being produced. We could, therefore, hypothesize: (i) that an elongated form of mRNA is generated and that it somehow ends up in the translation of a partially functioning normal protein; or (ii) that alternative splicing processes produce two forms of RNA , but that only one of them is able to translate a correct protein.

To verify these working hypotheses, we undertook a study on ectopic mRNA obtained from the only available tissue, peripheral blood. mRNA was obtained from the lymphocytes of 6/10 patients and from 40 healthy controls, following informed consent. cDNA, reverse transcribed (Reverse Transcription kit - Clontech, Palo Alto,

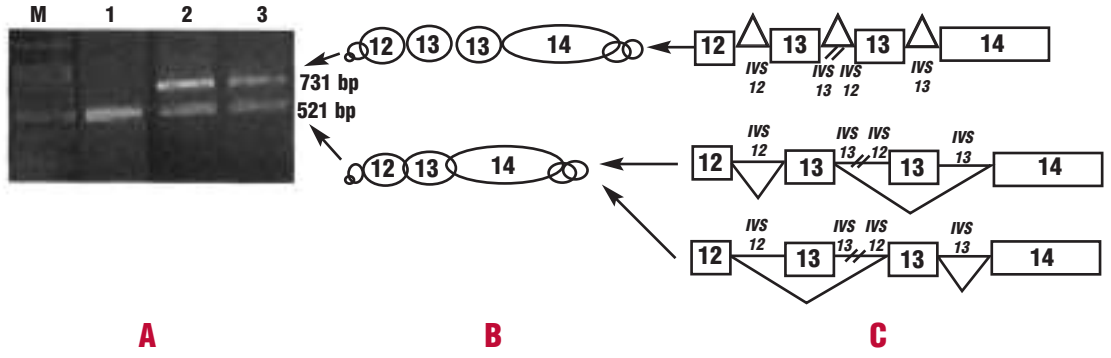


Figure 1. mRNA analysis and schematic representation of alternative splicing processes which lead to differently sized fragments. A. Analysis of ectopic mRNA amplified by RT-PCR. Five microliters from the second PCR amplification products from both normal and patient RNA were electrophoresed through a 2% ethidium bromide stained gel. Lane M: 100 bp DNA ladder; lane 1, mRNA from a normal control; lanes 2 and 3, mRNA from two different patients carrying the exon 13 duplication. Lengths of the normal (521bp) and mutated (731bp) fragments are shown. B. Partial schematic representation of the mRNA of the patients. C. box: exon; triangle: intron; // indicates the junction of part of intron 13 with a part of intron 12 as the result of the duplication. Schematic drawing representing the theoretical splicing processes which could lead to the elongated (731bp) and normal-sized (521bp) fragments. Upper part: splicing occurs within the newly created intron sequence (prediction score 0.89). Middle and lower parts: alternative splicing involving different donor and acceptor sites (see text) leads to exon 13 skipping.

CA, USA) from total RNA, was amplified under standard conditions with 50 pmol primers located at positions 1814 to 1835 (11F: 5'-TGACCCGCTATTACTC-TAGTTT-3') and 2659 to 2677 (14AR: 5'-GAAAAAGTCTCATATTTGGC-3'). Seminested PCR amplification (30 cycles: 94°C for 1 min, 55°C for 2 min, 72°C for 2 min) was performed using primer 11F and primer 14BR (5'ATGCCTCTGTTCCGAAAGTC3') sited at positions 2314 to 2333 ( GenBank, accession number M14113, *http://www.ncbi.nlm.nih.gov*). The results showed that besides the 521 bp product, which was the only mRNA fragment present in the healthy controls (Figure 1A, lane 1) an additional 731 bp fragment was detected in the RNA of HA patients (Figure 1A, lanes 2 and 3). Long and short fragments from the mRNA of HA patients, excised from the 2% agarose gel, purified (Ultrafree-DA Centrifugal Device- Millipore Corporation, Bedford, MA, USA), and sequenced using a fluorescent ABI Prism Big Dye terminator kit (Applied Biosystem, UK), showed that the aberrant-sized fragment (731 bp) contained two exons 13, duplicated in frame. Following the duplication, a new sequence was created whereby the first amino acid of the duplicated exon 13 was a glycine instead of the native serine. Sequencing of the normal-sized fragment confirmed that it contained one exon 13 both in the patients and in the normal controls.

From these experimental data we can assume that: (i) the residual FVIII coagulant activity derives from the 521 bp RNA fragment (Figure 1B); (ii) the normal-sized fragment is the result of two possible splice processes (Figure 1C) in which i) the donor sequence is the exon 12 one and the acceptor sequence is the duplicated exon 13 one; ii) the donor sequence is the first exon 13 one and the acceptor site is the exon 14 one. In both processes one exon 13 is skipped. We can also infer that the abnormal-sized RNA transcript (Figure 1B) originates from a splicing process which uses the original intron 13 donor site and the native intron 12 acceptor site. Both sequences are located within the new intron, which is formed by the junction of about 2500 bp of 5' intron 13 with about 700 bp of 3' intron 12 (Figure 1C, upper). The intron 12 branch does not move from its original location, i. e. -76 nucleotides from the 3' intron 12 end. The computer-assisted analysis for splice site prediction on genomic DNA (*http://www.fruitfly.org*) supports the assessment of this new splicing process, with an 0.89 splice prediction score.

Since we were not able to determine the FVIII antigen levels in any of our patients, we cannot comment on the translation product of the aberrant mRNA. It is well known that high levels of FVIII mRNA are present in tissues synthesizing FVIII protein, i.e. liver and kidney[3] and therefore, these should be the sources of mRNA to be used for correct and most precise quantitative studies. In fact, very low amounts of illegitimate mRNA from circulating peripheral blood do not properly reflect FVIII mRNA expression from the synthesizing cells.[4] However, if we assume that a protein is produced, it would contain the 70 additional residues that are codified by the duplicated exon and, on the basis of the residual FVIII:C, it is likely non-functional. To better understand the causes of the detrimental effects that the mutation has on the protein's function, we inspected the molecular model of the FVIII protein using the O' program[5] on a Silicon Graphics Octane R12K biprocessor and by secondary structure prediction run on the Network Protein Sequence Analysis.[6] The extra 70 amino acid residues may cause incorrect folding of the secondary structure of the FVIII protein. The addition of seven β strands may significantly change the physico-chemical properties of the protein's surface and, therefore, possible interactions of FVIII with other proteins could be affected.

To our knowledge, this is the first example of a mutation which interferes positively in HA with the splicing mechanism, since one of the two induced alternative splicing processes (exon skipping) *repairs* a genomic error.

*Maura Acquila,* Mirella Pasino,* Tiziana Lanza,* Angelo Claudio Molinari,* Camillo Rosano,° Maria Patrizia Bicocchi**

**Department of Haematology and Oncology, Thrombosis and Haemostasis Unit, Giannina Gaslini Institute, Genova, Italy; °Bioinformatics and Structural Proteomics National institute for Cancer Research (IST), Genova, Italy*

*Acknowledgments: the authors would like to thank Drs. Ulrich Pfeffer and Luca Anfosso for their contribution to the revision of this manuscript.*

*Funding: this work was supported in part by the Compagnia di S. Paolo and by Fondazione Carige.*

*Key words: factor VIII, hemophilia A, exon 13, duplication, skipping.*

*Correspondence: Mirella Pasino, Ph.D., Department of Hematology and Oncology, Thrombosis and Hemostasis Unit, G. Gaslini Institute, largo G. Gaslini 5, 16147 Genoa, Italy. Phone: international +39.010.5636277. Fax: international +39.010.386204. E-mail: mirellapasino@ospedale-gaslini.ge.it*

## References

1. Acquila M, Pasino M, Lanza T, Bottini F, Molinari AC, Bicocchi MP. Duplication of exon 13 causes one third of the cases of mild hemophilia A in northern Italy. Haematologica 2004;89:758-9.
2. Wu Q, Krainer AR. AT-AC pre-mRNA splicing mechanisms and conservation of minor introns in voltage-gated ion channel genes. Mol Cell Biol 1999;19:3225-36.
3. Hollestelle MJ, Thinnes T, Crain K, Stiko A, Kruijt JK, van Berkel TJ, et al. Tissue distribution of factor VIII gene expression in vivo-a closer look. Thromb Haemost 2001;86:855-61.
4. El-Maarri O, Herbiniaux U, Graw J, Schroder J, Terzic A, Watzka M, et al. Analysis of mRNA in hemophilia A patients with undetectable mutations reveals normal splicing in the factor VIII gene. J Thromb Haemost 2005;3:332-9.
5. Jones TA, Zou JY, Cowan SW, Kjeldgaard M. Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr A 1991;47:110-9.
6. Combet C, Blanchet C, Geourion C, Delage G. NPS@: network protein sequence analysis. Trends Biochem Sci 2000; 25:147-50.

*Disorders of Hemostasis*

## Factor XI deficiency with a novel homozygous mutation Trp599Arg near the C-terminal region

**We identified a novel mutation in an asymptomatic 72-year old Japanese woman with severe factor XI (FXI) deficiency. Sequence analysis showed a homozygous missense mutation Trp599Arg (g.234T→C according to Genbank accession number M20218). This residue belongs to a region conserved in human FXI and the FXI of several animals. Molecular modeling showed that the Trp599 residue is positioned in an alpha helix in the C-terminal region of the FXI molecule.**

haematologica 2005; 90:999-1001
(*http://www.haematologica.org/journal/2005/7/999.html*)

The factor XI gene (*F11*) (23 Kb in size) is located on chromosome 4q35, and contains 15 exons plus 14 introns.[1] To date, more than 40 different FXI gene lesions have been documented in the factor XI mutation database (*http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/119891.htm*). We identified a novel causative homozygotic mutation in a Japanese woman with severe factor XI (FXI) deficiency, which resulted in a Trp599 to Arg substitution near the carboxyl-terminal region of FXI. The proband was a 72-year old Japanese woman who consulted Iki public hospital after she had been bitten by a mamushi pit viper. She was immediately treated by serotherapy and coagulation tests were performed. Her platelet count was decreased for a time, but was within the normal range after a few hours. She had no bleeding tendency in that time. While results showed a remarkably prolonged activated partial thromboplastin time (89 s, reference range; 28-32s), her bleeding time, prothrombin time and fibrinogen levels were within normal ranges. No inhibitor of the intrinsic coagulation pathway was found in her plasma. Her parents were first cousins. After obtaining signed documents of informed consent, blood specimens were drawn from the proband and her son. FXI coagulant activity (FXI:C) was assayed by a one-stage method based on the activated partial thromboplastin time using FXI-deficient plasma. FXI antigen (FXI:Ag) concentrations were determined using goat anti-FXI-IgG (Cedarlane, Ontario, Canada), biotin labeled anti-FXI-IgG and peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark) by an in-house enzyme-linked immunosorbent assay (ELISA). Both FXI:C and FXI:Ag levels of the proband were below 1% of normal control values. Coagulation factors, apart from FXI, were within normal ranges. FXI:C and FXI:Ag for her son were 56.6% and 62.0%, respectively. Polymerase chain reaction (PCR) amplification of exons 1 through 15 of the *F11* gene was performed with the use of genomic DNA as a template, with primer and conditions as described previously.[2] Purified PCR products were directly sequenced using a *Taq* Dye Deoxy Terminator Sequencing Kit (Applied Biosystems, Foster City, USA). Sequence analysis showed a T→C mutation at codon 599 in exon 15 (g.234T→C according to Genbank accession number M20218), resulting in a substitution of Trp599 (TGG) by Arg (CGG) in the catalytic domain. This point mutation created an *Msp* 1 restriction endonuclease recognition site in the exon 15 nucleotide sequences. Results of an *Msp* 1 digestion indicated that the proband was homozygous for the mutation, while her son was heterozygous for the mutation (Figure 1). No *Msp* 1 restriction endonuclease sites were detected in any of exon 15 sequences from 50 normal Japanese individuals.

Trp599 belongs to a structurally conserved region present in human FXI (Genbank accession number NM-000128), rabbit FXI (AF395821), bovine FXI (NM028066) and mouse FXI sequences (BC019485) (Figure 2A). It is

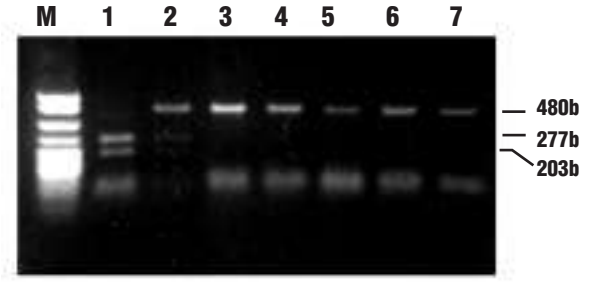


**Figure 1.** ***Msp 1*** **restriction analysis of the patient, her son and normal individuals. The normal individuals exhibit an undigested band at 480bp, while the variant gives digested bands at 203 and 277bp. This confirms the creation of an** ***Msp 1*** **cleavage site due to the altered sequence from CTGG to CCGG, and also demonstrates that the proband is homozygous for the observed mutation. Her son shows 480, 277 and 203bp bands, indicating that he is heterozygous for this mutation. PCR fragments spanning exon 15 were digested and analyzed on a 1.8% agarose gel. Lane 1, proband; lane 2, son; lane 3-7, normal individuals. The left lane contains DNA molecular weight markers.**