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Original scientific paper

IDENTIFICATION OF INTRON 1 AND INTRON 22 INVERSIONS OF FACTOR VIII GENE IN SERBIAN PATIENTS WITH HEMOPHILIA A

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Hemophilia A (HA) is a common X-linked recessive bleeding disease caused by mutations of FVIII gene. Inversion of intron 1 (inv1) and intron 22 (inv22) are recurrent mutations in severe HA, causing 50% of cases. Inv1 has been reported to occur in 2–5% and inv 22 in 45% of severe HA patients. Our objective was to determine, for the first time in Serbia, the frequency of inv1 and inv22 in a group of severe HA patients and to compare these data with those from other countries.

Study subjects were 50 HA patients, diagnosed and treated from April 2009 to June 2012 at Mother and Child Health Care Institute of Serbia "Dr Vukan Cupic" (IHS) and Institute for Child and Youth Health Care of Vojvodina (IHV).The presence of inv1 and inv22 was analyzed using Inverse shifting PCR (IS-PCR). Our results revealed that the frequencies of inv1 and inv22 in the cohort of Serbian patients were 6 % and 42% (34% of inv22 type I and 8% of inv22 type II) respectively . These frequencies were in line with those found in other populations. Carrier status analyses of 65 family members (mothers and sisters) showed the *de novo* inversion of intron 22 in one patient.

Genetic Counseling Units of IHS and IHV provide the adequate genetic advice to all HA affected patients and their family members.

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INTRODUCTION

Hemophilia A (HA) is a common X-linked recessive bleeding disease affecting approximately 1 in 5000 men (KLINGE *et al.,* 2002). It is caused by mutations in the FVIII gene (*F8*) (MIM#306700), that has 26 exons spanning 186-kb genomic DNA and mapped to the distal end of the long arm of X-chromosome (Xq28) (GITSCHIER *et al.,* 1984). These mutations can lead to FVIII protein deficiency or dysfunction. According to the residual plasma FVIII coagulant activity (FVIII: C), the disease can be divided into severe (<1%), moderate (1–5%) or mild (>5–40%) HA (WHITE *et al.,* 2001). The recurrent mutations in severe HA are the intron 1 and intron 22 inversions, which occur in 2–5% and 45% of this patients, respectively (BAGNALL *et al.,* 2002); (LAKICH *et al.,* 1993).

According to latest knowledge, intron 22 includes the presence of a bidirectional promoter that initiates transcription of two expressed genes (*F8A* and *F8B*). It is part of a GCrich sequence of 9.5 kb (*int22h-1*) that is duplicated at two positions towards the Xq-telomere (*int22h-2* and *int22h-3*). Sequencing of the human X chromosome has showed that *int22h-2* and *int22h-1* had the same orientation while *int22h-3* is in inverse orientation to them; *int22h-2* and *int22h-3* are a part of an imperfect palindrome with a central unique loop of 67,3 kb and arms of 50,5 kb (DE BRASI *et al.,* 2008) (Fig. 1.A.).

Intron 22 inversions (inv22) is a result of non-allelic meiotic homologous recombination between the *int22h-1* region within the *F8* locus and either *int22h-2* or *int22h-3*, in male germ cells (ROSSITER *et al.,* 1994). *Int22h-1* recombines with the most telomeric copy of *int22h* which is always inversely oriented to *int22h-1* and in most of the cases it is *int22h-3*. This *int22h-1/int22h-3* recombination lids to inv22 type I (Fig. 1A). In minor number of cases it was shown that inversion was a result of two recombination events. First one was a recombination between the arms of the palindrome *inv22h-2/ inv22h-3*, which has been established as a common non-deleterious inversion polymorphism. That event swaps the positions and orientations of *int22h-2* and put it at the most telomeric and inverse position to *inv22h-1*. The second recombination between *inv22h-1* and *inv22h-2* result in inv22 type II (BAGNALL *et al.,* 2005) (Fig. 1.B.).

Furthermore, it has been predicted that recombination between *int22h-1* with a milarly oriented copy of either *int22h, int22h-2* or *int22h-3* might be responsible for large deleterious deletions (Del22), and also presumably non-deleterious duplications (Dup22), as opposed to the classical inversions (BAGNALL *et al.,* 2006).

Inversion of intron 1 (inv1) of *F8* gene is another large molecular defect resulting in severe HA. The pathogenic mechanism associated with this inversion involves homologous recombination between a 1041 bp region of intron 1 (*int1h-1*) of the *F8* gene and inversely orientated an extragenic copy (*int1h-2*) of region approximately 140 kb telomeric to the *F8* gene (BAGNALL *et al.,* 2002; LAKICH *et al.,* 1993) (Fig1.C.).

The recombination between *int1h-1* and *int1h-2* repeats from sister chromatids or homologous chromatids and chromosomes, would result in dicentric chromosomes and acentric fragments and hence should not lead to viable embryos.

Both, the inv1 and inv22 prevent the formation of full-length *F8* messenger RNA (mRNA) and result in the absence of *F8* proteins leading to severe HA.

Other HA-causative mutations include a spectrum of nonsense, missense, splice-site mutations and small or large deletions / insertions that have been identified throughout the gene and compiled in international databases (HAMSTeRS, http://europium.csc.mrc.ac.uk). Due to its size and complexity, *F8* still challenges mutation characterization worldwide.

The goals of the present study were to assess the presence of inv22 and inv1 in the *F8* gene in Serbian severe HA patients and to compare these frequencies with published data from other populations. This study describes the first HA mutation series from Serbia.

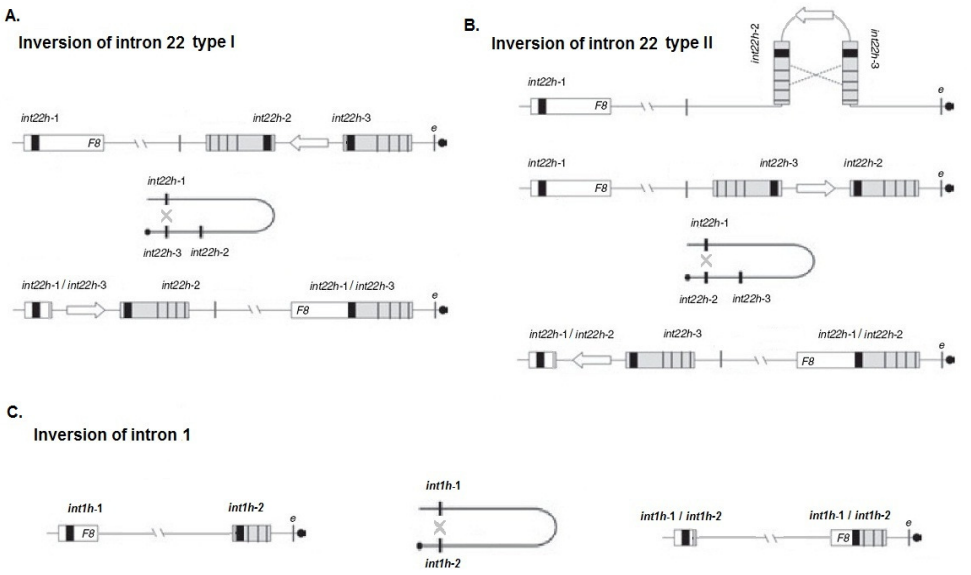


Fig. 1. Mechanism of non-allelic homologous recombination causing inv22 and inv1

A. Recombination between *int22h-1* and *int22h-3* in common palindrome *int22h-2/ int22h*-3 configuration, which cause inv22-type I.

B. Intra-chromosomal/chromatid homologous recombination which yield an inverted palindrome configuration *int22h-3/ int22h-2* and cause inv22-type II;

C. Recombination between *int1h-1* and *inv1h-2* which cause inv1.

MATERIALS AND METHODS

Patients

The study group includes 50 HA patients from 45 Serbian families who were diagnosed from April 2009 to June 2012 at Haemathooncology Departments of Mother and Child Health Care Institute of Serbia "Dr Vukan Cupic" (IHS) and Institute for Child and Youth Health Care of Vojvodina (IHV). Blood samples from patients with clinical diagnosis of HA were analysed

for the presence of inv 22 and inv1 of *F8* gene. Also, the 65 of family members (26 mothers and 39 sisters of patients) were under analyses for carrier status of mentioned inversions.

Methods

Clinical diagnosis of HA was verified by APTT (Activated Partial Thromboplastin Time) test and quantification of FVIII:C. Peripheral blood samples (5-10ml) were collected in EDTA-Na2 tubes and DNA purification was carried out by the standard salting-out method.

Inverse shifting-PCR (IS-PCR)

IS-PCR for detection of inv22 and inv1 was performed according to L .C.ROSSETTI protocol (ROSSETTI *et al.,* 2008). Genomic DNA (2 µg) was digested with 20 units of BclI according to the supplier's specifications (Promega) over 4 h in 50 lL. Digested DNA was isolated using phenol–chloroform and ethanol precipitation. DNA fragments were circularized with 3 units of T4 DNA Ligase (Invitrogen) in 400 µlat 15 °C overnight. Ligated samples were then treated with an equal volume of phenol:chloroform mixture, the aqueous phase was removed, and the ethanol-precipitated DNA recovered in 50 µL of distilled water. PCR was performed in reactions containing 3µl and 6 µl of circularized DNA for the analysis of Inv1 and Inv22, respectively, in the presence of 0.6 µM of each primer, 0.5 U of Taq DNA Polymerase (Promega) and additional standard PCR reagents in a total volume of 25 µL. Thermocycling involved 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 56°Cfor 1 min and extension at 72 °Cfor1.5 min; cycling was preceded by 94°Cfor 2 min, and followed by 5 min at 72 °C.

IS-PCR products were analyzed on ethidium bromide stained 1.5–2% agarose gel electrophoresis and photographed.

Statistical analysis

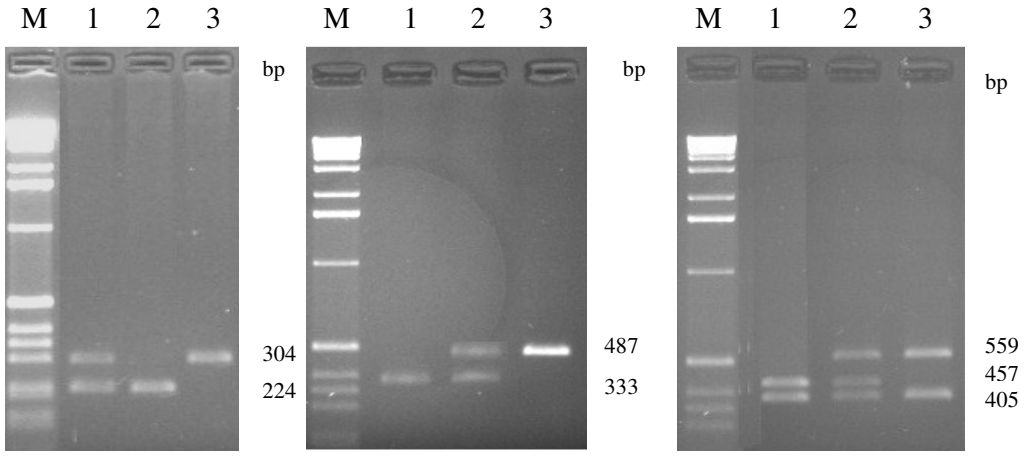
We performed the statistical analysis in order to compare the frequencies of inv22 and inv1 in FVIII gene in Serbian HA patients with similar published data from other countries. Person χ^2 test and Fisher exact test (two- tailed) were used depending on the values of results (program Statistica 7).

RESULTS

In our cohort of 50 unrelated patients with severe HA, 24/50 (48%) were found to have the inversion. The inv22 was detected in 21/50 (42%) of patients and in 3/50 (6%) the inv1 was revealed (Fig 2.).

Four of 21 (4/21) patients with inv22 had inv22 type II (19%) and the rest of patients (17/21) was positive for inv22 type I (80,9%%) (Tab1.). In the present series of 50 patients, the frequency of inv22 type II was 8% (4/50) and 34% (17/50) for inv22 typeI. In 3/50 (6%) patients inv1 was found (Fig 2.).

Carrier status analysis showed the presence of inversion in 25 mothers and 11 sisters of HA patients (Fig 2.). All analyzed mothers of hemophilic sons were carriers of the inversions, except in one case. According to these results in one HA patient inv22 type 1 occurred de *novo*.



A. Inv1 diagnostic test A. Inv22 diagnostic test C. Inv22 complementary test

Fig 2. The analysis of the IS-PCR products by standard agarose gel electrophoresis.

A. Inv1 diagnostic test: (1) inv1 carrier; (2) inv1 hemophiliac; (3) non-inv1 individual and M indicates a marker of 100 bp ladder.

B. Inv22 diagnostic test: (1) inv22 type 1 hemophiliac; (2) inv22 type 1 carrier; (3)non-inv22 individual and M indicates a marker of 100 bp ladder.

C. Inv22 complementary test for full characterization of all possible *int22h*-related rearrangements (i.e. inversions, deletions and duplications depicted in inv22 diagnostic test): (1) inv22 type 1 hemophiliac; (2) inv22 type 1 carrier; (3)non-inv22 individual and M indicates a marker of 100 bp ladder.

Tab.1. Frequency of inv22 and inv1 in Serbian patients with sever HA

<table>
<tr><td>N° of analyzed patients</td><td colspan="3">Positive for inversion</td><td>Negative for inversion</td></tr>
<tr><td rowspan="4">50(100%)</td><td colspan="2">Inv 22</td><td>Inv1</td><td rowspan="4"></td></tr>
<tr><td>Type I</td><td>Type II</td><td rowspan="3"></td></tr>
<tr><td>17(34%)</td><td>4(8%)</td></tr>
<tr><td colspan="2">21(42%)</td><td>3(6%)</td></tr>
<tr><td colspan="3">24(48%)</td><td>26(52%)</td></tr>
</table>

DISCUSSION

The frequency of the recurrent inv 22 and inv1 in severe hemophilia A patients is about 50% (for inv22 40-50%, for inv1 2-5%), according to literature, without significant differences between populations. (BAGNALL *et al.,* 2002; LAKICH *et al.,* 1993). In our study, for the first time performed in Serbian population, the frequency of inversions was in the same range (48%).

The detected frequency of inv22 (42%), type I (34%) and type II (8%) in severe HA Serbian patients is similar to those observed in other populations. Comparison with the related studies made at Germany (OLDENBURG *et al.,* 2006), Italy (ACQUILA *et al.,* 2003), Spain (CASAÑA *et al.,* 2008), UK (BAGNALL *et al.,* 2002), Mexico (MANTILLA-CAPACHO *et al.,* 2007), Argentina (ROSSETTI *et al.,* 2004), Brazil (LEIRIA *et al.,* 2009), India (FARIDI *et al.,* 2012) and China (XUE *et al.,* 2010) did not show statistically significant differences (Tab. 2.).Our results are also consistent with an international consortium study where 43% of 2093 unrelated severe HA patients were positive for inv22 (35% type I and 7% type II) (ANTONARAKIS *et al.,*1995).

The suggested prevalence of inv1 in literature is 2-5% in general population of hemophilia A patients, what correspond with our results of 6%. When we compared our data with those from 9 other countries we did not observe the significant difference, except for the samples from Mexico (Tab.2.). The incidence of inv1 in this country was 0%. The larger studies from these countries are required before the validation of any ethnic differences.

All published studies, so far, including ours, verified that the inv1 occurred at about one tenth the frequency of the inv22. This may largely be due to the size of the *int1h* repeats that are 9-fold smaller than *int22h* (1041 versus 9503bp). Additional reason could be the presence of only one exstrogenic copy of *int1h*, whereas two *int22h* copies. The similarity between copies of *int1h* is very high (99.9%), as it is between repeats of *int22h*, so the degree of similarity should not be responsible for the difference in frequency of above mentioned inversions (BAGNALL *et al.,* 2002).

According to carrier status investigation, mother of hemophilic son has an approximately 80% chance of being a carrier when her son is the first affected individual in the family, and this chance is even higher (98%) if only inv22 is considered (LEUER *et al.,* 2001). These results may be due to somatic mosaicism which predominantly occurs in a female members of family. Germline mosaicism is rare. Intrachromosomal recombination among the homologous regions of intron 22 is thought to be almost exclusively of meiotic origin, arising predominantly in male germ cells, so this pathogenic mechanism would argue against a somatic origin of an intron 22 inversion during early embryogenesis. However, one instance of somatic mosaicism with this mutation type has been observed (OLDENBURG *et al.,* 2000). Somatic mosaicism of the intron 22 inversion caused by a post-zygotic de novo mutation would imply that this mutation is not, as suggested before, exclusively restricted to meiotic cell divisions, but it may also occur during mitotic cell divisions either in germ cells or in somatic cells.

In our study, were 25 mothers had been analyzed for carrier status of inv22 and inv1, we found only one case with non carrier mother. So, the inv22 in one HA patient we considered as *de novo* mutation. The IS –PCR method which we used for inv22 detection was able to reveal minimal mosaic composition of 5% (ROSSETTI *et al.,* 2008). According to that, inv22 in our patient may be due to the presence of low percent of mosaicism in mother's somatic cells or to some post-zigotic event in patient's somatic cells.

In 45 families with HA we also detected 11 sisters who were carriers of recurrent inversions.

Genetic counseling units of HIS and IHV provide the adequate genetic advice to all HA affected patients and their family members.

Tabela 2. Prevalence of inv22 and inv1in different ethnic populations

Ethnic population (Reference)	Frequency of inv22	%	Significance	Frequency of inv1	%	Significance
Europe						
Serbia (this article)	21/50	42	/	3/50	6	/
Germany (OLDENBURG *et al.*,2006)	339/753	45	0,68	19/753	2,5	0,15
Italy (ACQUILA *et al.*, 2003)	39/93	42	1	3/54	5	0,66
Spain (P CASAÑA *et al.*, 2008)	42/102	41	0,92	4/134	3	0,66
UK (BAGNALL *et al.*, 2002)	94/209	45	0,71	10/209	5	0,99
America						
Mexico (MANTILLA-CAPACHO *et al.*, 2007)	14/31	45	0,77	0/65	0	0,08
Argentina (ROSSETTI *et al.*, 2004)	25/64	39	0,75	1/64	1,5	0,31
Brazil (LEIRIA *et al.*, 2009)	46/107	43	0,92	3/107	2,8	0,40
Asia						
India (FARIDI *et al.*, 2012)	35/80	44	0,84	3/80	3,8	0,68
China (XUE *et al.*, 2010)	57/148	39	0,66	3/148	2	0,34

CONCLUSION

The first study performed on Serbian hemophilia A patients showed that the frequency of inv22 (42%) and inv1 (6%) was in line with similar published data from other countries. The further molecular analyses should be performed on HA patients lacking inv22 and inv1, in order to detect the other underlying mutations, which cause this disease.

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IDENTIFIKACIJA INVERZIJE INTRONA 1 I INTRONA 22 GENA U GENU ZA FAKTOR KOAGULACIJE VIII KOD PACIJENATA OBOLELIH OD HEMOFILIJE A IZ SRBIJE

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Izvod

Hemofilija A (HA) je X-vezano recesivno oboljenje koje nastaje kao posledica mutacija u genu za faktor koagulacije VIII (*F8*). Do sada je identifikovan veliki broj različitih tipova mutacija u *F8* genu, od kojih su najučestalije inverzija introna 1 (inv1) i inverzija introna 22 (inv22). Ove mutacije su prisutne kod 50% obolelih od teškog oblika HA; inv1 je otkrivena kod 2–5%, a inv22 (inv22) kod 45% pacijenata. Cilj ove studije je bio da se odredi učestalost pomenutih inverzija u grupi HA pacijenata iz Srbije, jer do sada ovakvi podaci nisu publikovani.

Analiza je urađena na uzorku od 50 obolelih kojima je u period od aprila 2009. do juna 2012. HA dijagnostikovana na Institutu za zdravstvenu zastitu majke i deteta Srbije, „Dr Vukan Čupić" (IMD) i Institutu za zdravstvenu zaštitu dece i omladine Vojvodine (IZZZDIO). Za detekciju inv1 i inv22 korišćena je metoda inverznog PCRa (IS-PCR). Rezultati su pokazli da u analiziranom uzorku HA pacijenata iz Srbije učestalost inv1 iznosi 6%, a inv22 42% (34% inv22 tip I, 8% inv22 tip II). Ovi rezultati se slažu sa objavljenim rezultatima sličnih studija iz drugih zemalja. Analiza za određivanje statusa nosioca inverzija urađena je kod 65 članova porodica obolelih (majke i sestre) i ona je pokazala prisustvo *de novo* inv22 kod jednog pacijenta.

Svi oboleli od HA, kao i članovi njihovih porodica, dobili su odgovarajući genetički savet u okviru Genetičkog savetovališta IMDa i IZZZDIOa.

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