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Skewed X chromosome inactivation in fraternal female twins results in moderately severe and mild haemophilia B

（偏った X 染色体不活性化に伴う二卵性双生児の女性血友病 B）

名古屋大学大学院医学系研究科

医療技術学専攻

（指導：　小嶋　哲人　教授）

奥村　薫

Summary

Female carriers of haemophilia B are usually asymptomatic; however, the disease resulting from different pathophysiological mechanisms has rarely been documented in females. In this study, we investigated the mechanisms responsible for haemophilia B in fraternal female twins. We sequenced the factor IX gene (*F9*) of the propositus, her father, a severe haemophilia B patient, and the other family members. X chromosome inactivation was assessed by the methylation-sensitive *Hpa*II-PCR assay using X-linked polymorphisms in human phosphoglycerate kinase 1 gene (*PGK1*) and glutamate receptor ionotropic AMPA 3 gene (*GRIA3*). The twins were found to be heterozygotes with a nonsense mutation (p.Arg384X) inherited from their father. The propositus, more severely affected twin, exhibited a significantly higher percentage of inactivation in the maternally derived X chromosome carrying a normal *F9*. The other twin also showed a skewed maternal X inactivation, resulting in a patient with mild haemophilia B. Thus, the degree of skewing of maternal X inactivation is closely correlated with the coagulation parameters and the clinical phenotypes of the twins. Furthermore, we identified a crossing-over in the Xq25–26 region of the maternal X chromosome of the

more severely affected twin. This crossing-over was absent in the other twin, consistent with their fraternal state. Differently skewed X inactivation in the fraternal female twins might cause moderately severe and mild haemophilia B phenotypes, respectively.

Introduction

Haemophilia B is an X-linked recessive bleeding disorder caused by a deficiency or functional defect in the coagulation factor IX (FIX) with an incidence of 1:25,000–30,000 male births [1, 2]. It is a clinically heterogeneous disorder in which bleeding severity is related to the quantity of coagulant FIX activity (FIX:C), and is classified as severe (<1%), moderately severe (1≤ to <5%) or mild (5≤ to <30%) based on the FIX:C. FIX is a vitamin K-dependent plasma protein and is important in the early phase of blood clotting.

The human FIX is encoded by the FIX gene (*F9*), which is 34-kb in length comprising 8 exons and 7 introns, and is located on the X chromosome (Xq27.1–27.2). It is synthesized by hepatocytes as a 461 amino acid precursor with a 46 amino acid signal peptide, which is cleaved off prior to its secretion into plasma as a mature peptide (415 amino acids). Plasma FIX is a single chain glycoprotein with a molecular weight of approximately 57 kDa, with 5 important functional domains; the N-terminal Gla domain, the aromatic amino acid stack domain, two EGF-like domains, the activation peptide domain and the C-terminal catalytic domain [2-4].

Female carriers of haemophilia B are usually asymptomatic because the X chromosome is randomly inactivated with an approximately equal proportion of the 2 populations of somatic cells [5, 6]. However, in rare cases, a skewed X chromosome inactivation could result in a symptomatic female in whom the normal X chromosome is predominantly inactivated [7, 8]. In rare instances, structural X chromosome abnormalities such as translocations, iso-X chromosomes or others also cause haemophilia B in females [9-13].

Here, we have described fraternal twin girls carrying the FIX nonsense mutation inherited from their father, associated with differently skewed inactivation of the maternally derived normal X chromosome, causing moderately severe and mild haemophilia B phenotypes, respectively.

Patients, Material and Methods

Patients and samples

The propositus was a 1-year-old girl suffering from a subaponeurotic cephalohematoma following a head injury. She was diagnosed with moderately severe haemophilia B (FIX:C = 2.3%) and successfully treated with FIX concentrates (Fig. 1). Her fraternal twin sister was also diagnosed with mild haemophilia B (FIX:C = 24.6%). Their father was a severe haemophilia B patient (FIX:C<1.0%) and the mother had normal plasma FIX:C (88.9%). Cytogenetic analysis of the propositus showed a normal female karyotype (46, XX) without any structural abnormality. The study was approved by the Ethics Committee of the Nagoya University School of Medicine. After obtaining informed consents, citrated blood samples were obtained from the twins and their parents. From each sample, genomic DNA was isolated from the peripheral leukocytes by phenol extraction as described previously [14].

Identification of F9 mutation

The 8 exons and exon-intron boundaries of the *F9* were amplified by polymerase chain reaction (PCR) using the gene-specific primers listed in Table 1. The PCR products

were analyzed by direct sequencing using the BigDye Terminator Cycle Sequencing Kit and the ABI Prism 310 Genetic Analyzer according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) as described previously [15]. We also analyzed the DNA samples by PCR-mediated *Taq*I restriction fragment length polymorphism (RFLP) to confirm the identified mutation. Thus, DNA fragments of the *F9* (exon 8) were amplified by PCR as described above, treated with *Taq*I, and then analyzed by 2% agarose gel electrophoresis.

Southern blot analysis

DNA of the family members was subjected to *Apa*LI Southern blot and *Nco*I Southern blot analyses for *F9* as described previously [14].

Assessment of X chromosome inactivation by methylation-sensitive HpaII-PCR assay

X inactivation was assessed by the methylation-sensitive *Hpa*II-PCR assay using an X-linked human phosphoglycerate kinase 1 gene (*PGK1)* polymorphism as described previously [16]. The genomic DNA samples were pretreated with or without

methylation-sensitive *Hpa*II (New England BioLabs, Bervery, MA, USA), and then subjected to PCR-mediated *Bst*XI RFLP analysis for the *PGK1*. The PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide, and the amount of each product was evaluated by the NIH image version 1.62 (http://rsb.info.nih.gov/nih-image/).

We also analyzed X chromosome inactivation patterns at the X-linked glutamate receptor ionotropic AMPA 3 gene (*GRIA3*) as described previously [17]. Genomic DNA samples were treated with or without methylation-sensitive *Hpa*II, and subjected to PCR amplification with specific fluorescent primers (Table 1). The PCR products were then analyzed by GeneScan software on ABI Prism 310 Genetic Analyzer.

X Chromosome haplotype analysis

To analyze the X chromosome haplotype of the family members, we used polymorphic markers, such as monoamine oxidase A gene (*MAOA*, Xp11.23) promoter [18], androgen receptor gene (*AR*, Xq11–12) [19], phosphoglycerate kinase 1 gene (*PGK1*, Xq13.3) [16], glutamate receptor ionotropic AMPA 3 gene (*GRIA3*, Xq25–26) [17], FIX gene (*F9* -793, Xq27.1–2) [20] and Fragile X mental retardation gene 1 (*FMR1*, Xq27.3)

[16]. We also analyzed short tandem repeat [13] polymorphisms, such as reference SNP IDs: rs3223331 (Xq22), rs3220745 (Xq24) and rs3220178 (Xq26).

Results and discussion

Haemophilia B occurs primarily in males. Normally, female carriers of haemophilia B do not exhibit a phenotypic manifestation of the bleeding disorder. However, there are several different mechanisms that could lead to the phenotypic expression of very low FIX:C levels in females [7]. In this study, we investigated the molecular basis of moderately severe and mild haemophilia B phenotypes in fraternal twin girls, in order to elucidate the genetic mechanisms responsible for female haemophilia B.

First, we analyzed *F9* genes of the family members by direct sequencing and found a C-T transition at nucleotide position 1150 of the coding sequence (c.1150C>T). This nonsense mutation (p.Arg384X), previously reported as FIX New York [21], was responsible for the immature termination of the FIX protein synthesis in their father, a severe haemophilia B patient. This mutation was also detected in both the twins in heterozygous state (Fig. 1), which was confirmed by the *Taq*I PCR-RFLP analysis (data not shown). The variant FIX molecule did not have normal activity, consistent with the undetectable FIX:C level of their father. However, the heterozygous mutation in the twins indicated inconsistency in their moderately severe and mild haemophilia B phenotypes.

*Apa*LI or *Nco*I Southern blot analysis performed for *F9* in the twin girls demonstrated no altered migration pattern, suggesting the absence of gross gene abnormality in their *F9* (data not shown). Furthermore, the heterozygous state of the *F9* c.1150C>T mutation in the propositus indicated that she would not have a large gene deletion of the *F9* in her maternal allele, which could be missed in PCR analysis.

We analyzed a possibility of skewed inactivation of the maternally derived normal X chromosome in the twins. To assess the heterozygosity for a G/A polymorphism in intron 1 of the *PGK1* [16], we performed PCR-mediated *Bst*XI-RFLP analysis for the family and found that both the twins were positive. Thus, the *Bst*XI-RFLP analysis for the twins showed 2 distinct bands, which indicated the G allele from the father (372-bp) and the A allele from the mother (468-bp) (Fig. 2). After *Hpa*II digestion of the template DNA from the peripheral blood, the PCR-mediated *Bst*XI-RFLP analysis for the propositus twin girl showed an extremely unbalanced amplification towards the maternal allele (99:1), indicating a severely skewed inactivation of the maternally derived normal X chromosome. Contrastingly, the other twin showed only a moderately skewed amplification towards the maternal allele (65:35), which was consistent with her mild haemophilia B phenotype. We also analyzed X chromosome inactivation patterns at the X-linked *GRIA3* [17] and

observed similar skewed inactivation results of the maternally derived X chromosome in the fraternal twins (Fig. 3). Therefore, the difference in the severity of the haemophilia B phenotype closely correlated with the degree of skewing of X inactivation.

During the survey of informative markers for X inactivation assay along the X chromosome, we found a crossing-over in the maternal X chromosome at Xq25–26 region of the more affected twin (Fig. 1). This was absent in the other twin. This difference between the twins was consistent with their fraternal state. In the case of monozygotic (MZ) twins, Côté and Gyftodimou described that a review of twin discordance and an attempt at explaining some of the data leads one to hypothesize the impossibility for crossed X chromosomes to undergo efficient inactivation [22]. In fraternal twins, however, extreme skewed inactivation of crossed X chromosomes can result from single gene mutations that affect cell survival or growth [23]. Interestingly, it has been reported that X chromosome haplotype analysis suggests the presence of a locus for the familial skewed X inactivation in chromosome Xq25 most likely controlling the X chromosome choice in X inactivation or cell proliferation [24]. Therefore, although clarification of a precise break point of the X chromosome is necessary to confirm it, it might be possible that the crossing-over in the propositus X chromosome would disturb a gene function affecting cell

proliferation, leading to a selection towards the skewed X inactivation.

In conclusion, differently skewed inactivation of the maternal X chromosome in the fraternal female twins, with an inherited *F9* nonsense mutation in the paternally derived X chromosome, might cause moderately severe and mild haemophilia B phenotypes, respectively.

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Table 1. Primers used in this study.

	Primers 5' to 3'	Annealing temperature, °C	Product size, bp
FIX sequence primers			
FIX Exon1U	AATCAGACTAACTGGACCAC		
FIX Exon1L	TATCTAAAAGGCAAGCATAC	50	536
FIX Exon2&3U	ATGATGTTTTCTTTTTTGCT		
FIX Exon2&3L	GGTTGGACTGATCTTTCTG	50	553
FIX Exon4U	TTCTAAGCAGTTTACGTGCC		
FIX Exon4L	GTAGCTTCTTGAACTCATATCC	52	331
FIX Exon5U	CCCCCAATGTATATTTGACC		
FIX Exon5L	CCGTCCTTATACTAGAAGCC	50	606
FIX Exon6U	AATACTGATGGGCCTGCT		
FIX Exon6L	AACTTGCCTAAATACTTCTCAC	50	445
FIX Exon7U	CCAATATTTTGCCTATTCCT		
FIX Exon7L	CTTCTGGTATGGAAATGGCT	50	457
FIX Exon8U-1	TGTGTATGTGAAATACTGTTTG		
FIX Exon8L-1	TTATAGATGGTGAACTTTGTAGA	60	388
FIX Exon8U-2	TTGGATCTGGCTATGTAAGT		
FIX Exon8L-2	AGTTAGTGAGAGGCCCTG	60	407
PGK 1			
PGK1 Up	CTGTGCTCTGTCGCA AACCT		
PGK1 Lw	CTCCTGAAGTTAAATCAACATCC	52	468
GRIA 3			
GRIA 3 Up	GGAGTAACCACTAATGGCCCTC		562
GRIA 3 Up 6-FAM	GCGGAGAGAGTCTCAGATTGTC		182
GRIA 3 Lw	TTTGCCAAAGCGATTTCTGT	68	

Figure legends

Fig. 1. Pedigree and haplotype analysis of the family

The square (□) and circle (◯) symbols denote male and female, respectively. Solid symbols indicate the presence of *F9* nonsense mutation (p.Arg338X: c.1151C>T). Solid arrow (↘) indicates the propositus. Values (%) below the symbols represent individual FIX:Cs. Haplotypes of the X chromosomes are shown in the boxes, which have number of the repeat or individual nucleotide in each locus. Underline represents the *F9* mutation in this family. Asterisks (*) are loci used for X chromosome inactivation analysis. Open arrow (⇦) indicates a crossing-over in the X chromosome at Xq25-26 region of the propositus.

Fig. 2. X chromosome inactivation analysis at the *PGK* locus

A: Scheme of PCR amplification after methylation sensitive *Hpa*II digestion in the *PGK1* locus. The symbol ((Me)) indicates a methylation site on an *Hpa*II cleavage site.

B: *Bst*XI-RFLP at *PGK1* locus. The paternal derived G allele is detected as 372-bp and 96-bp fragments, whereas the maternal derived A allele appears as a 468-bp fragment.

C: +; *Hpa*II digested DNA, —; undigested DNA, I.1; father (severe haemophilia B), I.2; mother (not informative), II.1; propositus (extremely skewed X inactivation), II.2; twin sister (mild skewed X inactivation).

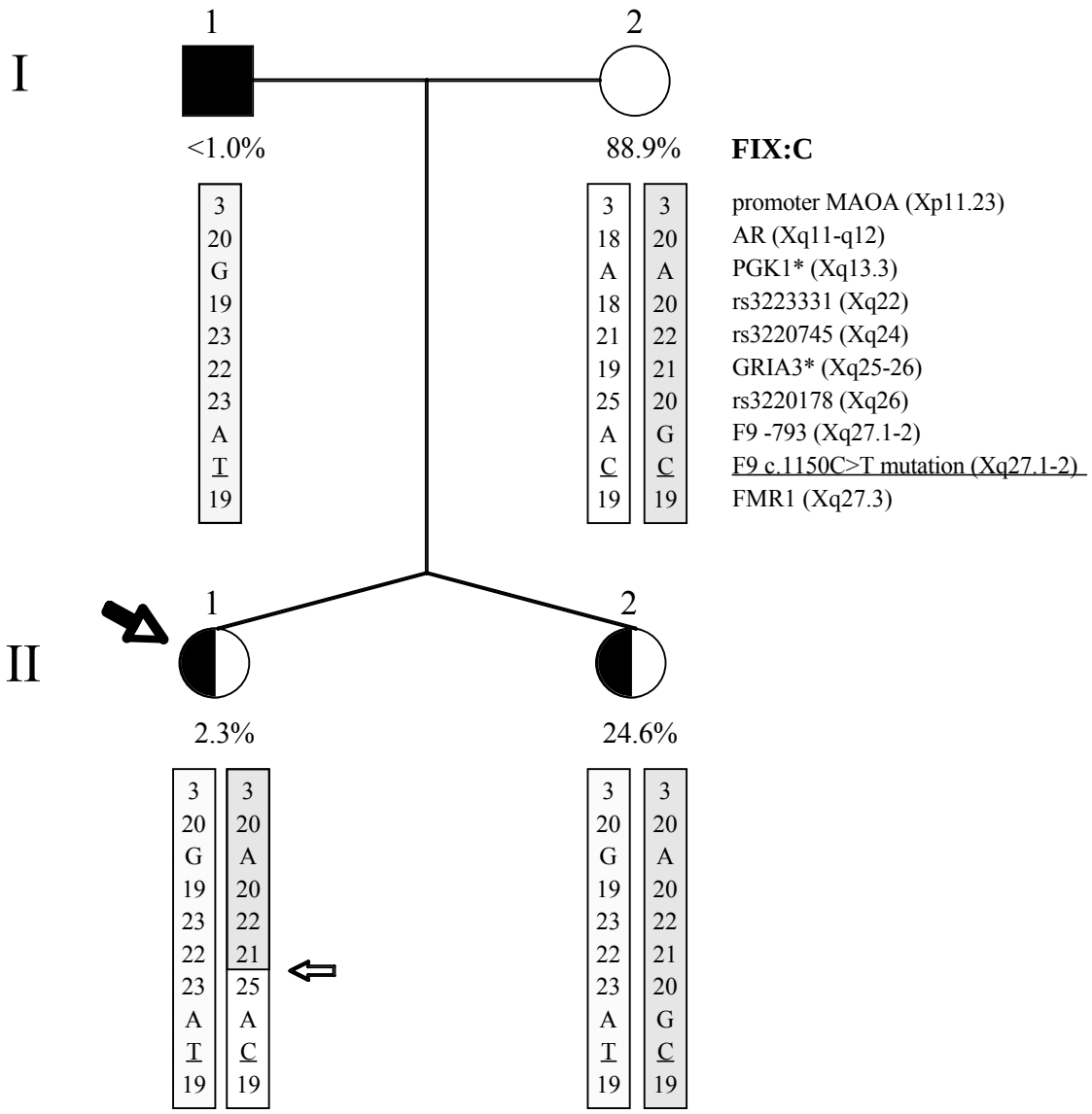
After *Hpa*II digestion of the DNA, PCR-mediated *Bst*XI-RFLP analysis for the propositus (II.1) showed an extremely unbalanced amplification towards the maternal A allele (99:1), indicating a severely skewed inactivation of the maternal X chromosome. Contrastingly, the other twin (II.2) showed only a moderately skewed amplification towards the maternal A allele (65:35).

Fig. 3. X chromosome inactivation analysis at the *GRIA3* locus

A: Scheme of nested PCR amplifications with specific primers (1st: GRIA 3 Up vs GRIA 3 Lw, 2nd: GRIA 3 Up 6-FAM vs GRIA 3 Lw) flanking both *Hpa*II site and polymorphic microsatellite TC repeats in the 5' region of *GRIA3* locus.

B: The upper (*Hpa*II−) and the lower (*Hpa*II+) represent the PCR amplifications without and with *Hpa*II digestion, respectively. The propositus (II.1) is heterozygous at this locus, inheriting the allele 'm2' (19 TC repeats) from her mother, and the allele 'p' (20 TC repeats) from her father. The signal of the paternal allele 'p' decreased remarkably in the PCR with *Hpa*II digestion (↓) compared with that of the maternal allele 'm2', suggesting that a severely skewed inactivation of the maternal X chromosome.

Fig. 1.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C
19
promoter MAOA (Xp11.23)
AR (Xq11-q12)
PGK1* (Xq13.3)
rs3223331 (Xq22)
rs3220745 (Xq24)
GRIA3* (Xq25-26)
rs3220178 (Xq26)
F9 -793 (Xq27.1-2)
F9 c.1150C>T mutation (Xq27.1-2)
FMR1 (Xq27.3)
II
1
2
2.3%
24.6%
3
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G
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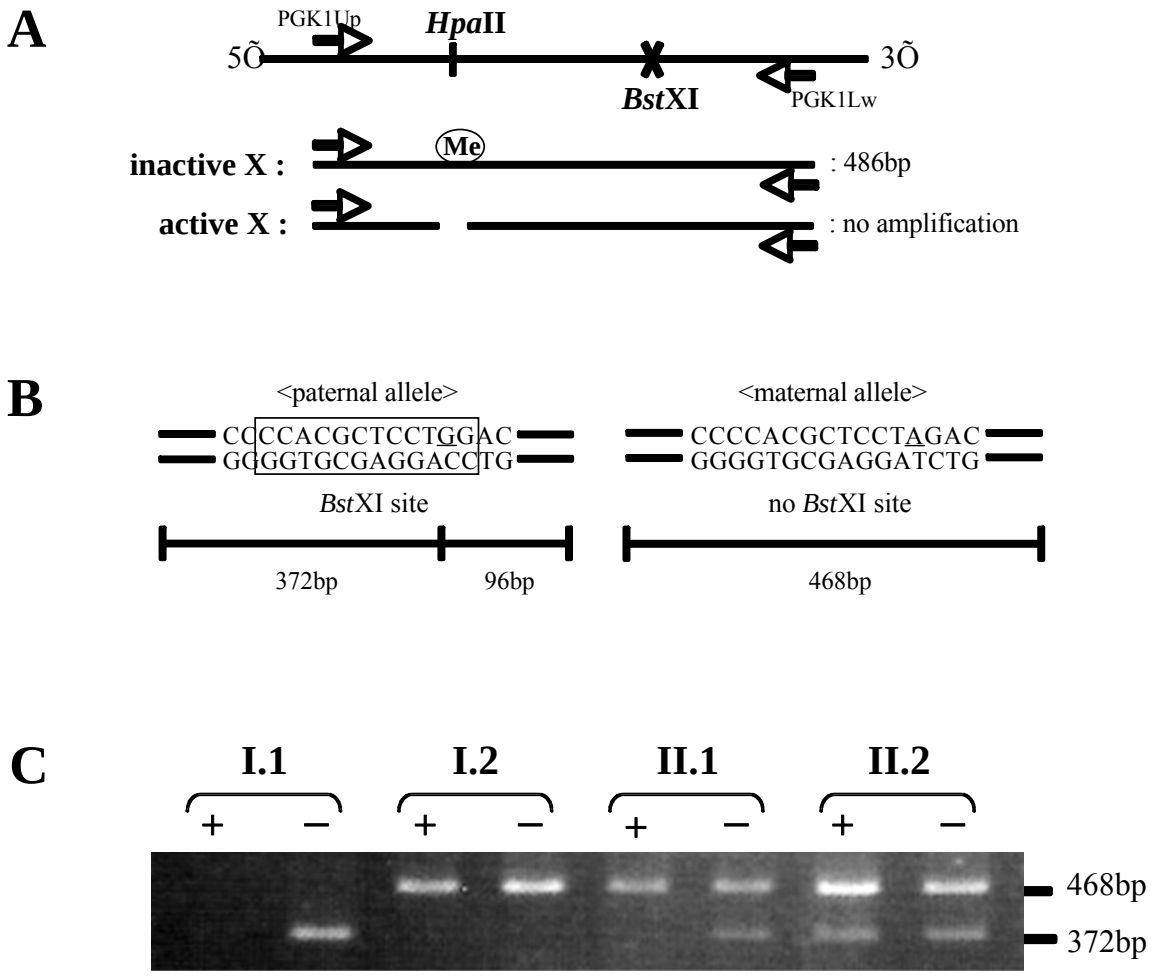
Fig. 2.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BstXI
PGK1Lw
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active X :
: no amplification
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BstXI site
372bp
96bp
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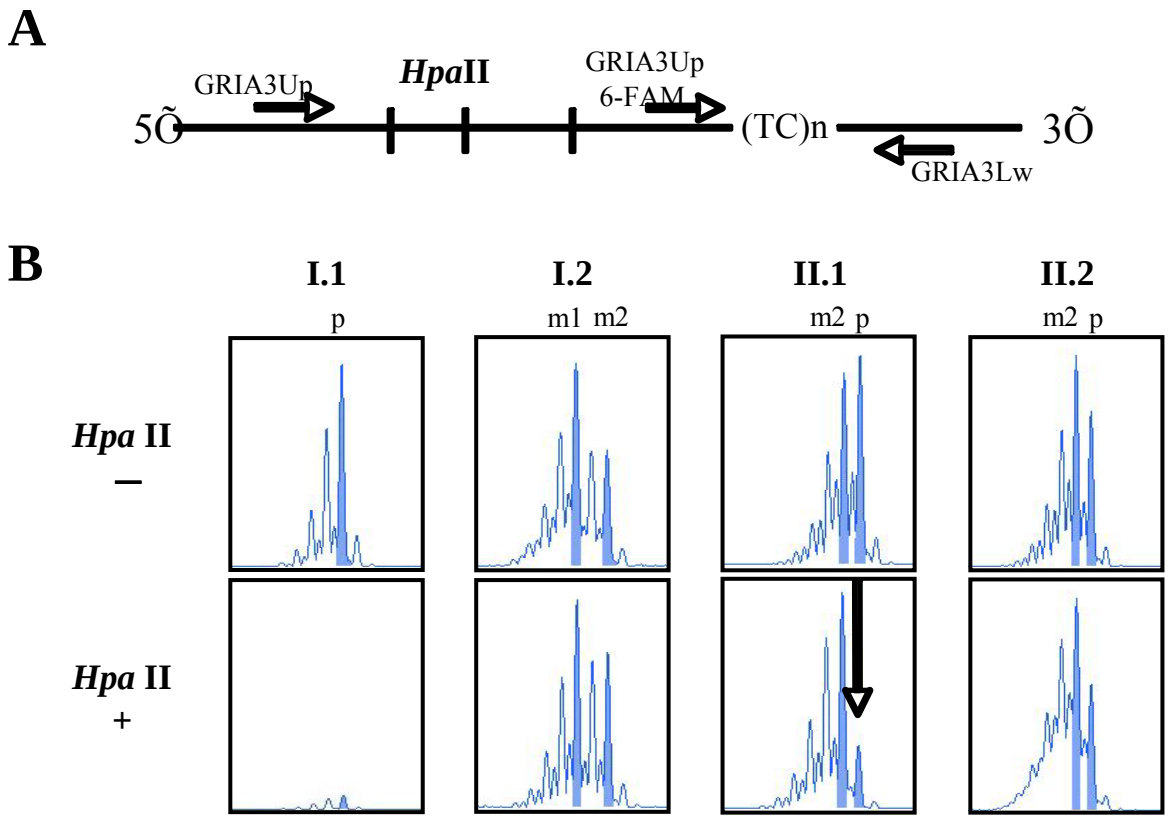
372bp

Fig. 3.


A
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6-FAM
5Õ
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m2 p
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Hpa II
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