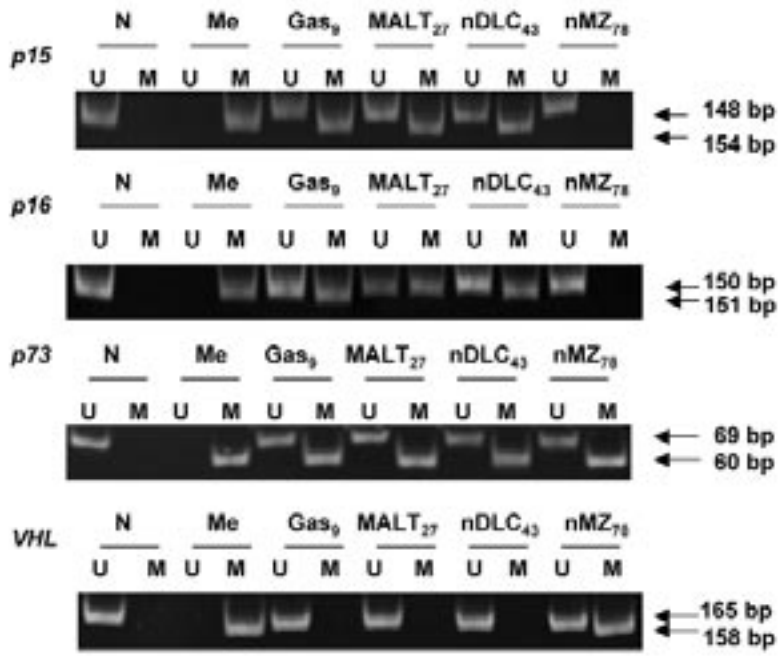

Figure 1. MSP of *p15*, *p16*, *p73* and *VHL* genes. N: normal control DNA, showing positive amplification with the unmethylated primers (U) but not with the methylated (M) primers; Me: methylated DNA, showing positive amplification with the M but not the U primers; Gas_9: gastric diffuse large B-cell lymphoma (case 9); $MALT_{27}$: gastric MALT lymphoma (case 27); $nDLC_{43}$: nodal diffuse large B-cell lymphoma (case 43); nMZ_{78}: nodal marginal zone B-cell lymphoma (case 78). For the *p15* gene, Gas_9, $MALT_{27}$ and $nDLC_{43}$ were methylated, but nMZ_{78} was not. For the *p16* gene, Gas_9, $MALT_{27}$ and $nDLC_{43}$ were methylated, but nMZ_{78} was not. For the *p73* gene, Gas_9, $MALT_{27}$ and $nDLC_{43}$ and nMZ_{78} were all methylated. For the *VHL* gene, only the nMZ_{78} was methylated.

shown that *p73* was very frequently methylated in natural killer cell lymphomas.[5] In lymphoma cell lines, methylation of *p73* correlated with down-regulation of the *p73* protein, and promoter demethylation led to re-expression of *p73*.[5,7] Interestingly, our results also showed that *p73* was frequently methylated in DLBC lymphoma in the stomach but not the lymph node. The significance of this in gastric lymphomagenesis merits further investigation.

Finally, the significance of gene methylation in MALT/MZBC and DLBC lymphomagenesis will need to studied by demonstrating that methylation-induced gene suppression contributes to cellular growth dysregulation or transformation.

Maggie K.L. Fung, Wing Y. Au, Raymond Liang, Gopesh Srivastava, Yok L. Kwong

University Department of Medicine, Queen Mary Hospital, Hong Kong

Correspondence: Dr Yok Lam Kwong, University Department of Medicine, Professorial Block, Queen Mary Hospital, Pokfulam Road, Hong Kong. Phone: international +852.28554597. Fax: international + 852.29741165. Email: ylkwong@hkucc.hku.hk

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Rapid genotyping of *Xba*I and *Msp*I DNA polymorphisms of the human factor VIII gene: estimation of their combined heterozygosity in the Argentinean population

In hemophilia A, indirect analysis using factor VIII gene polymorphisms is particularly valuable to obtain rapid information for genetic counseling. Herein, we describe an alternative route to investigate two intron 22 DNA polymorphisms (*Xba*I and *Msp*I) using an intragenic 12kb-long amplimer. The estimated heterozygosities on 37 haplotypes from the Argentinean population were *Xba*I (49%), *Msp*I (50%), and combined *Xba*I+*Msp*I (63%).

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Hemophilia A (HA) is an X-linked inherited bleeding disorder due to deficiency in the coagulation factor VIII (FVIII).

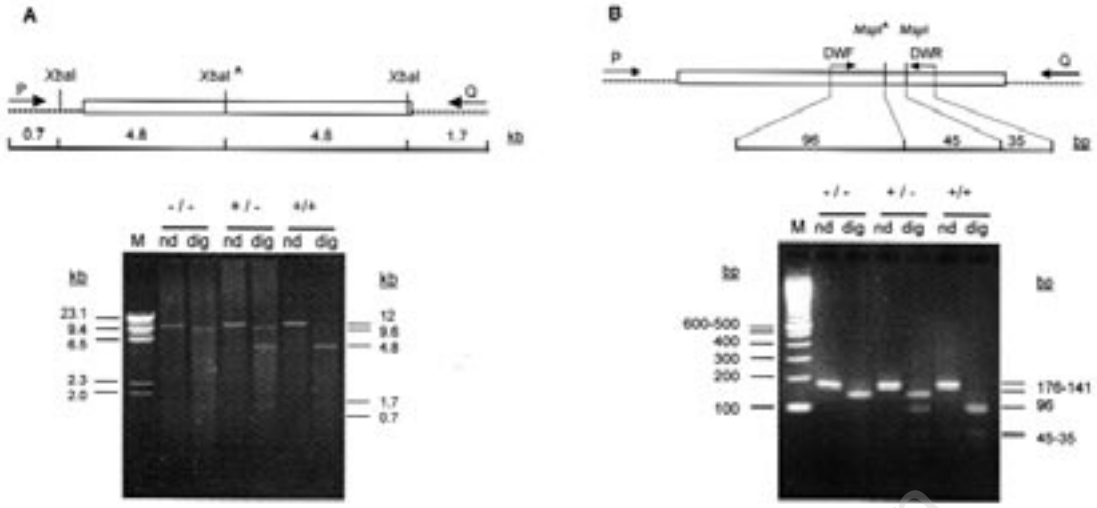

Figure 1. Analysis of *Xba*I A (X) and *Msp*I A (M) restriction fragment length polymorphisms (RFLPs). (a) X-RFLP analysis. Primers P and Q[1] amplify 12kb from *int22h*-1 sequences. Optimal conditions for PQ LD-PCR were: 600 ng of genomic DNA, 50 mM Tris/HCl pH 9.2, 1.75 mM $MgCl_2$, 5% (v/v) dimethyl sulphoxide, 16 mM $(NH_4)_2SO_4$, 0.6 μM of primers P and Q and 210 μM dGTP, 140 μM 7-deaza dGTP, 350 μM dA- dT- and dCTP and 2U *Taq*/*Pwo* DNA polymerase mixture (Expand long template DNA polymerases, Roche) in a volume of 20 μL. Three-temperature PCR were used for thermo-cycling.[5,6] PCR products (5-10 μL) were digested using 5U of enzyme in 15 μL of total volume under the conditions recommended by the manufacturer (Promega or Gibco BRL). The figure shows a diagram of the PQ LD-PCR with *Xba*I restriction map of the product. The 12kb-product from *int22h*-1 contains the polymorphic *Xba*I A and two constant sites that provide a control for *Xba*I digestion (restriction maps: GenBank entries AF062514, H86011 and H86012). Agarose gel electrophoresis (0.8%) of the PQ LD-PCR product digested with *Xba*I. Each pair of lanes contains undigested (nd) PCR product and corresponding digested (dig) product. Restriction patterns of homozygous [-/-], heterozygous [+/-] and homozygous [+/+] alleles are indicated in each case. Lane M corresponds to DNA size standard (λ *Hind*III). Due to ethidium-bromide staining, 4.8kb-long molecules will stain half as intensely as equal amounts of molecules of 9.6kb. In this method for X-genotyping, the 9.6kb-segment is cleaved into two fragments of 4.8kb, therefore the signals that represent the alleles [+] and [-] from X [+/-] heterozygotes show equivalent fluorescent intensities. (b) *Msp*I A RFLP analysis. Scheme of the nested PCR approach: PQ LD-PCR (1st round) and DWF+DWR PCR (2nd round) with the relevant *Msp*I restriction map. Aliquots (1 μL of 1:200 dilution) from the PQ LD-PCR product were subjected to nested PCR and analyzed as described Bowen *et al*.[3] The 176bp DW-PCR product from *int22h*-1 contains the polymorphic *Msp*I A and a constant site which provides a control for *Msp*I digestion. Agarose gel electrophoresis (2%) of the DW PCR product digested with *Msp*I. Lane M corresponds to a DNA size standard (100 bp ladder). Relevant signal sizes are indicated.

Except for intron 22 (Inv22) and intron 1 inversions, responsible for approximately one half of severe phenotypes, direct HA mutation diagnosis is time-consuming and labor-intensive because of the size and complexity of the gene. Currently, the Inv22 can be diagnosed by a single-tube long-distance polymerase chain reaction (LD-PCR).[1] When the causative mutation has not been established in an affected family, and it is not possible to analyze the entire FVIII gene, carrier and prenatal diagnosis relies upon gene tracking using intragenic DNA polymorphisms. Among these, *Xba*I A (X) and *Msp*I A (M) restriction fragment length polymorphisms (RFLP) showed high informativity in almost all the ethnic groups studied so far.[2,3] X and M reside within a 9.5kb-long sequence (*int22h-1*) of the FVIII gene intron 22, which is repeated twice extragenically (*int22h*-2 and -3) at Xq28.[4]

We propose an alternative method to genotype X and M. This method is based on the amplification of a 12kb-PCR product, using primers P and Q designed by Liu *et al*.,[1] which spans *int22h*-1 plus its intron-22 flanking sequences.

Two sets of experiments were performed to adjust the PQ LD-PCR conditions: primer concentration (0.2, 0.4, 0.6, 0.8 and 1.0 μM) and thermo-cycling type (two, three-temperature and sub-cycling PCR).[5] Optimal conditions for PQ LD-PCR are detailed in Figure 1A.

The restriction patterns predicted for all X genotypes were verified using six DNA samples from healthy Argentinean blood donors: 2 males (X [-] and [+]), 2 females ([-/+]), and 2 females ([-/-] and [+/+]) previously genotyped by validated methods.[6] These last 2 individuals with genotype XbaI A [+] (and [+/+]) were also genotyped for the extragenic RFLPs XbaI BC [- -] (and [-/- -/-]).[7] The complete absence of [-] signal observed in their X*ba*I restriction analysis proves the specificity of the 12kb-amplimer for X, therefore for int22h-1 and consequently for M.

Specific M genotyping is achieved by nested PCR: aliquots from the PQ LD-PCR product were subjected to a second round of PCR using primers DW,[3] to generate an intragenic-specific 176bp-PCR product which presents all the requirements for reliable M genotyping (Figure 1B). The same series of six individuals used for X genotyping were employed to investigate M-genotypes by nested PCR using the 12kb-product as a new specific substrate for the second round. Both methods, the validated one[3] and the modification presented here, gave matching results.

Short amplimers from heterozygous-RFLP samples could undergo free association of their *Watson* and *Crick* strands to form the duplex. This phenomenon would produce equivalent amounts of each ([Watson:Crick], [+:+] and [-:-]) homoduplex and ([+:-] and [-:+]) heteroduplex. Among these four species only [+:+] homoduplex is cleaved. Although this effect does not interfere with M-genotyping, Figure 1b shows the 3:1 ([-]:[+]) signal-intensity ratio. Long-size amplimers are not

Table 1. Frequencies and heterozygosities of *Xba*I / *Msp*I / *Bcl*I haplotypes in the Argentinean population.

Haplotype			Frequency		Marker/s	Heterozygosity		
X	M	B	No.	%		[2]O (%)	[3]E (%)	O/E
+	+	+	17	46	X	49		
+	+	–	0	0	M	50		
+	–	+	2	5	B	47		
+	–	–	0	0	[1]X+M	63	74	0.85
–	+	+	3	8	[1]M+B	58	73	0.79
–	+	–	1	3	[1]B+X	58	72	0.80
–	–	+	1	3	X+M+B	65	86	0.75
–	–	–	13	35				
Total			37	100				

X, M and B code for markers XbaI A, MspI A and BclI (intron-18), respectively. [1]Significant allelic associations (Fisher's exact test p< 0.01). [2]Observed heterozygosity calculated from individual haplotype frequencies supposing Hardy-Weinberg equilibrium (H-W) ($\sum {}^2pq \times 100$). [3]Expected heterozygosities calculated supposing free allelic association of markers.This represents the estimation of the combined heterozygosity in absence of linkage disequilibrium.

prone to suffer this effect.

This technique for streamline X and M-genotyping is rapid, simple and robust and hence particularly useful for providing information for carrier and prenatal diagnosis when there is no time to perform direct mutation-detection through the entire FVIII gene. Besides, due to the access of primers, reagents and enzymes, this approach can be easily mounted in all diagnostic laboratories that currently investigate the Inv22 by LD-PCR.[1]

As for other X-linked inherited disorders, on a statistical basis one third of the causative mutations of severe HA are lost per generation. This high turnover of severe mutations makes it unlikely that there is a strong linkage *disequilibrium* between one of them and particular alleles of DNA polymorphisms.[8] These features allow the estimation of the parameters associated with severe HA using those obtained from the healthy population. Accordingly, DNA samples from thirty-seven Argentinean males were genotyped for X, M and intron-18 BclI RFLP (B) in order to estimate single-*locus* and multi-*locus* heterozygosities. B-genotyping was performed using single PCR-digestion.[9] X, M and B Hardy-Weinberg heterozygosities for each marker by itself were estimated to be 49%, 50% and 47%, respectively (Table 1). The haplotype frequencies allowed the calculation of both expected (supposing complete linkage *equilibrium*) and observed combined heterozygosities. Although all three possible marker pairs showed significant allelic associations, the most effective combination was observed with X+M (63%) (observed/expected, 0.85) and the addition of B did not appreciably improve this figure (X+M+B, 65%) (Table 1). The X+M combined heterozygosity in the Argentinean population is similar to that reported from a Caucasian population (59%).[3]

Sequential investigation of the Inv22 (42%),[10] X (28%) and M (8%) potentially provide informative markers for most (78%) families affected by severe HA. In conclusion, the data presented here indicate that this procedure would improve the provision of rapid information for genetic counseling in HA families.

Carlos D.De Brasi, Liliana C. Rossetti, Irene B. Larripa

Departamento de Genética, Instituto de Investigaciones Hematológicas Mariano R. Castex, Academia Nacional de Medicina, Buenos Aires, Argentina

Correspondence: Carlos De Brasi, MD, PhD, Departamento de Genética, Instituto de Investigaciones Hematológicas Mariano R. Castex, Academia Nacional de Medicina, Pacheco de Melo 3081(1425), Buenos Aires, Argentina.
Phone: international +5411.48058803. Ext: 241.
Fax: international +5411.48039475.
E-mail: cdebrasi@hematologia.anm.edu.ar

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