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Carrier detection and prenatal diagnosis of hemophilia A in a Korean population by PCR-based analysis of the *BclI*/intron 18 and St14 VNTR polymorphisms

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Abstract We have undertaken this study to identify the usefulness of polymerase chain reaction (PCR)-based analysis of DNA polymorphisms in the *Bcl*I/intron 18 and St14 variable number of tandem repeats (VNTR) loci for carrier detection and prenatal diagnosis of hemophilia A in the Korean population. We have analyzed these polymorphisms in members of 105 unrelated Korean families with severe hemophilia A. The observed heterozygosity rates for the *Bcl*I/intron 18 and St14 VNTR polymorphisms were 21.0% and 71.3%, respectively. The *Bcl*I/intron 18 polymorphism was less informative in Koreans when compared with Caucasians and Japanese. The allele frequencies for St14 VNTR in Koreans were different from those in Caucasians. Compared with Caucasians, there was a markedly higher occurrence of low molecular weight alleles in Koreans. The observed heterozygosity for the St14 VNTR polymorphism in combination with the *Bcl*I/intron 18 polymorphism was 81.9%. These two polymorphisms were applied to determine the carrier status of 107 women from 65 unrelated families, and to assess fetal status in 37 pregnancies. So far, we have experienced one case of misdiagnosis of carriership. Our study demonstrated that the PCR-based analysis of the *Bcl*I/intron 18 and St14 VNTR polymorphisms was useful in the carrier detection and prenatal diagnosis of hemophilia A in the Korean population.

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Introduction

Carrier detection and prenatal diagnosis of hemophilia A has been greatly improved by molecular diagnosis through direct and indirect gene analysis. Although methods for the direct detection of pathogenic mutations such as factor VIII intron 22 gene inversions have been developed recently (Lakich et al. 1993; Pieneman et al. 1995), indirect gene analysis using DNA polymorphisms still plays an important role in the carrier detection and prenatal diagnosis of hemophilia A. Many polymorphic markers within or near the factor VIII gene have been reported as being useful for the carrier detection and prenatal diagnosis of hemophilia A: *Bcl*I/intron 18 polymorphism (Gitschier et al. 1985), *Xba*I/intron 22 polymorphism (Wion et al. 1986), intron 13/22 dinucleotide repeat polymorphisms (Lalloz et al. 1991), and extragenic St14 variable number of tandem repeats (VNTR) located at 4 cM from the factor VIII gene (Oberle et al. 1985; Richards et al. 1991). The polymerase chain reaction (PCR) has made DNA polymorphism analysis simpler and more rapid for clinical applications than Southern blot analysis, and it can be applied in the analysis of most polymorphisms linked to the factor VIII gene. However, the analysis of the *Xba*I polymorphism by PCR has not been reliable, because the homologous non-factor VIII region on X chromosome is also coamplified (Kogan et al. 1987).

The allele frequencies in a given factor VIII restriction fragment length polymorphism (RFLP) differ significantly in various ethnic groups (Chan et al. 1988; Graham et al. 1990). Thus, the clinical usefulness of a DNA polymorphism should be determined according to ethnicity. In this study, we examined whether PCR-based analysis of DNA polymorphisms in the *Bcl*I/intron 18 and St14 VNTR loci was useful for carrier detection and prenatal diagnosis of hemophilia A in the Korean population.

Subjects and methods

Subjects

*Bcl*I/intron 18 and St14 VNTR polymorphisms were analyzed in 182 unrelated subjects (105 mothers and 77 fathers) in 105 unrelated families with severe hemophilia A. These two polymorphisms were utilized to determine carrier status in 107 women from 65 unrelated families. We performed prenatal diagnosis in 40 pregnancies. All subjects were Korean, and were enrolled in this study after informed consent was obtained.

Sample preparations

Genomic DNA was extracted from leukocytes obtained from the subjects' peripheral blood by one of two methods: (1) a modified phenol/chloroform extraction procedure (Sambrook et al. 1989), or (2) with Wizard genomic DNA purification kits (Promega, Madison, WI, USA). Factor VIII coagulant (FVIII:C) levels were measured by activated partial thromboplastin time, using a chromogenic assay (Dade Behring, Newark, NJ, USA) with Electra 1000C (MLA; New York, NY, USA). Chorion villous samples were obtained by the transvaginal route under ultrasound guidance at 10 to 12 weeks of gestation. Transabdominal amniocentesis was performed between 16 and 18 weeks of gestation. For the prenatal diagnosis, DNA was extracted from either chorionic villi or amniocytes. Both chromosomal analysis and PCR with Y-chromosome-specific primer sets (Kogan et al. 1987) were used for fetal sexing.

Factor VIII gene analysis

The *Bcl*I/intron 18 and St14 VNTR polymorphisms were analyzed by PCR. PCR was performed as described previously (Sarkar et al. 1989; Richards et al. 1991), with minor modifications. PCR was performed by adding 0.1–0.5 μg of DNA to 50 μl PCR reaction mixture (final concentration; 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 50 μM each dNTP, and 0.5 μM of each primer and 1 U of Taq polymerase).

Oligonucleotides were as follows: primers 5′-ATGTGT TCACTGTACGA-3′ and 5′-AATATCTTGGGATGG AC-3′ for the *Bcl*I region (Sarkar et al. 1989) and primers 5′-GGCATGTCATCACTTCTCTCATGTT-3′ and 5′-CACCACTGCCCTCACGTCACTT-3′ for the St14 VNTR region (Richards et al. 1991).

After 1 min at 94°C for initial denaturation, 30 (St14 VNTR) or 35 (*Bcl*I) cycles of PCR were performed (denaturation, 30 s at 94°C; annealing, 1 min at 55°C; extension, 2 min at 72°C) with an automated thermal cycler (PE Applied Biosystems, Forster City, CA, USA). For the *Bcl*I polymorphism, 10 μl of amplified product was digested with *Bcl*I restriction enzyme at 50°C for 2–4 h and submitted to 3%–4% agarose gel electrophoresis and stained with ethidium bromide. For St14 VNTR, amplified products underwent 1.5% agarose gel electrophoresis and were stained with ethidium bromide.

Cordocentesis

For prenatal diagnosis, cordocentesis was performed at 18–24 weeks of gestation in the following instances, with parental consent: (1) when the linkage analysis was not informative in pregnancies with a male fetus; (2) to confirm the disease status in an affected male fetus diagnosed by molecular analysis; and (3) when the parents strongly desired to confirm fetal status by fetal blood coagulation study although molecular diagnosis suggested an unaffected fetus.

Results

Factor VIII activities in obligate carriers

Factor VIII activity was measured in 83 obligate carriers of severe hemophilia A. Factor VIII activity ranged from 7.9% to 125%, with a median value of 44.9%.

Heterozygosity rate of each RFLP

The expected rate of heterozygosity of the *Bcl*I/intron 18 RFLP, calculated from allele frequencies of 84.7% and 15.3%, was 25.9% (Table 1). Twenty-two of the 105 unrelated women (21.0%) studied showed a heterozygous pattern for the *Bcl*I/intron 18 RFLP.

The PCR for St14 VNTR in 182 unrelated individuals

Table 1. Allele frequencies of two polymorphisms linked to the factor VIII gene in the Korean population

Polymorphisms	Number of X chromosomes	Allele frequencies (%)[a]		Heterozygosity rate (%)	
		+	−	Observed[b]	Expected
*Bcl*I	287	84.7	15.3	21.0	25.9
St14 VNTR	287	15 alleles		71.3	77.8

VNTR, Variable number of tandem repeat
[a] The symbols + and – indicate the presence or absence of a restriction site, respectively
[b] Observed rate for 105 unrelated females

(105 females and 77 males) of 105 families revealed 15 different alleles, ranging from 700 bp to 2890 bp (Fig. 1), and the most frequent alleles in the Korean population were 700 bp (42.2%), 1290 bp (13.6%), and 1350 bp (9.8%) in decreasing order of frequency (Table 2). From the frequencies of the 15 alleles, an expected heterozygosity rate of 77.8% was obtained. St14 VNTR RFLP showed heterozygous patterns in 77 of the 105 (73.3%) unrelated women studied. With the combination of *Bcl*I/intron 18 RFLP and ST14 VNTR, 86 of the 105 women (81.9%) showed the heterozygous pattern.

Carrier detection

The determination of carrier status was undertaken in 107 at-risk women from 65 unrelated families, in whom linkage analysis with *Bcl*I/intron 18 RFLP or St14 VNTR was possible. Of 54 women from 35 families with a known family history of hemophilia A, 27 women were diagnosed as carriers, and 27 women as non-carriers. Of 53 women from 30 families with sporadic hemophilia, carriership was excluded in 24 women, and 29 women were diagnosed as having a possibility of carriership.

Since the beginning of this study in 1994, we have experienced, so far, one case of misdiagnosis of carriership among 107 subjects assessed. The patient had a known family history, and was diagnosed as a non-carrier by analysis of the St14 VNTR polymorphism. However, the *Bcl*I polymorphism was uninformative in that subject. We established her carrier status after her daughter's delivery of a hemophiliac. Repeated molecular analysis of the St14 VNTR polymorphism in that family showed the same result as in the initial study. Therefore, we suspect that the misdiagnosis may have been caused by recombination between the St14 VNTR region and the factor VIII gene.

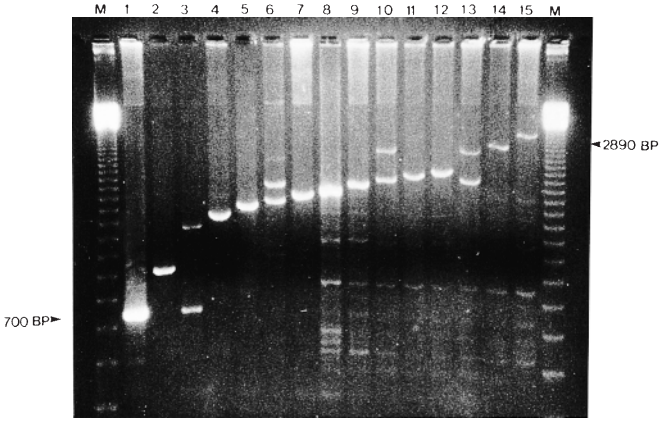

Fig. 1. Alleles of St14 variable number of tandem repeats (VNTR) region amplified by polymerase chain reaction (PCR). PCR Products underwent 1.5% agarose gel electrophoresis. Fifteen alleles were found, ranging from 700 bp (*lane 1*) to 2890 bp (*lane 15*). The details are shown in Table 2. *Lane M*, 123 bp ladder

Table 2. Allele frequencies of the St14 VNTR polymorphism in Koreans

No. of alleles	Alleles (bp)	Frequency	Percentage in the population
1	700	121	42.2
2	840	2	0.7
3	1170	6	2.1
4	1290	39	13.6
5	1350	28	9.8
6	1420	23	8.0
7	1480	7	2.4
8	1540	14	4.9
9	1600	13	4.5
10	1720	15	5.2
11	1780	3	1.0
12	1850	4	1.4
13	2280	2	0.7
14	2460	9	3.1
15	2890	1	0.3
Total		287	100

Prenatal diagnosis

We attempted 37 prenatal diagnoses in 32 women, using the two polymorphisms described above (Table 3): 10 women were obligate carriers; 7 were diagnosed carriers with a family history; and 15 women were suspected carriers without a family history. Fetal sex was determined by chromosomal analysis of either amniocytes or chorionic villi.

Of 20 prenatal diagnoses in the 17 women with a family history of hemophilia A, 10 fetuses were diagnosed as normal males, 5 as normal females, and 4 as carriers. One fetus was diagnosed as an affected male by molecular analysis, which was confirmed by measuring factor VIII activity in fetal blood by cordocentesis (case 1 in Table 4). Of 17 prenatal diagnoses in the 15 women without a family history, 7 fetuses were diagnosed as normal males, 1 as a normal female, and 6 as carriers. Three fetuses were diagnosed as affected males; 2 (cases 2 and 3 in Table 4) diagnoses were confirmed by cordocentesis, and for the third fetus, the mother elected to terminate the pregnancy without confirmation by cordocentesis. In seven unaffected males diagnosed by molecular analysis (cases 4–10 in Table 4) the diagnoses were also confirmed by cordocentesis. Of the fetuses subjected to prenatal diagnosis performed above, no infant or child has been found, until now, to have any hemorrhagic manifestation or abnormal factor VIII activity after birth.

We performed prenatal diagnosis by cordocentesis in three additional male fetuses (cases 11–13 in Table 4). The carriership of the mothers could not be determined by linkage analysis. Factor VIII activity of fetal blood in these three subjects was within the normal range.

Four male fetuses (cases 14–17 in Table 4) whose mothers were diagnosed as non-carriers by molecular analysis underwent cordocentesis at the request of the parents, and showed normal factor VIII activity in cord blood.

Table 3. Details of prenatal diagnoses performed by linkage analysis

Case no.	Carrier status of mother	Samples obtained	RFLP employed	Molecular diagnosis	Cordocentesis (case no. in Table 4)
Known family history					
1	Obligate	CV	St14	Carrier	No
2	Obligate	CV	St14	Normal female	No
3	Obligate	CV	St14	Normal female	No
4	Obligate	CV	St14 and *Bcl*I	Normal male	No
5	Obligate	CV	St14	Carrier	No
6	Obligate	CV	St14	Normal male	No
7	Obligate	AM	*Bcl*I	Normal male	Yes (case 7)
8	Obligate	CV	St14	Normal male	No
9	Obligate	CV	St14	Carrier	No
10	Obligate	CV	St14	Normal male	Yes (case 10)
11	Obligate	AM	St14	Normal male	No
12	Obligate	CV	*Bcl*I	Normal female	No
13	Diagnosed	AM	St14 and *Bcl*I	Normal male	No
14	Diagnosed	CV	St14 and *Bcl*I	Carrier	No
15	Diagnosed	AM	St14	Normal female	No
16	Diagnosed	CV	St14	Normal male	No
17	Diagnosed	AM	St14	Affected male	Yes (case 1)
18	Diagnosed	CV	St14	Normal female	No
19	Diagnosed	CV	St14	Normal male	No
20	Diagnosed	CV	St14	Normal male	Yes (case 9)
With sporadic hemophilia					
1	Putative	CV	St14 and *Bcl*I	Carrier	No
2	Putative	CV	St14 and *Bcl*I	Affected male	Yes (case 2)
3	Putative	CV	St14 and *Bcl*I	Normal female	No
4	Putative	CV	St14 and *Bcl*I	Carrier	No
5	Putative	AM	St14	Affected male	Yes (case 3)
6	Putative	AM	St14	Normal male	Yes (case 4)
7	Putative	CV	St14	Normal male	Yes (case 5)
8	Putative	CV	St14	Carrier	No
9	Putative	CV	St14 and *Bcl*I	Affected Male	No
10	Putative	CV	St14	Normal male	Yes (case 6)
11	Putative	CV	St14	Normal male	No
12	Putative	CV	St14	Carrier	No
13	Probable	CV	St14	Normal male	No
14	Probable	CV	St14 and *Bcl*I	Normal male	No
15	Probable	CV	St14	Carrier	No
16	Probable	CV	St14	Carrier	No
17	Probable	CV	St14	Normal male	Yes (case 8)

RFLP, Restriction fragment length polymorphism; diagnosed, carrier diagnosed by linkage analysis; putative, mother of a patient with hemophilia A; probable, carrier suggested by linkage analysis; AM, amniocytes; CV, chorionic villi

Table 4. Factor VIII activity in the cord blood of male fetuses, determined by cordocentesis

Case no.	Gestational age at cordocentesis (weeks)	Factor VIII activity (%)	Molecular diagnosis	Final outcome
1	21	5	Affected	Terminated
2	20	<0.7	Affected	Terminated
3	22	0.8	Affected	Terminated
4	20	37	Unaffected	Delivered
5	20	76	Unaffected	Delivered
6	22	85	Unaffected	Delivered
7	21	40	Unaffected	Delivered
8	22	50	Unaffected	Delivered
9	22	45	Unaffected	Ongoing
10	21	62	Unaffected	Ongoing
11	21	25	Uninformative	Delivered
12	18	43	Uninformative	Delivered
13	22	29	Uninformative	Delivered
14	20	125	Unaffected[a]	Delivered
15	24	31	Unaffected[a]	Delivered
16	20	21	Unaffected[a]	Ongoing
17	22	45	Unaffected[a]	Ongoing

[a] The mother was diagnosed as a non-carrier by molecular analysis

Discussion

Our study showed that the *Bcl*I/intron 18 and St14 VNTR polymorphisms were useful in the carrier detection and prenatal diagnosis of hemophilia A in the Korean population, and that the combination of these two polymorphisms made the molecular diagnosis of hemophilia A possible in about 80% of the Korean subjects investigated.

The allele frequencies and the heterozygosity rate for the *Bcl*I polymorphism in Koreans were somewhat different from those found in Caucasians or Japanese. The heterozygosity rate for the *Bcl*I polymorphism has been reported to be 36%–42% in Caucasians (Gitschier et al. 1985; Salle et al. 1990), 42% in Japanese (Suehiro et al. 1988), and 14%–30% in Chinese (Chan et al. 1988; Salle et al. 1990). Thus, the *Bcl*I polymorphism was less informative in Koreans (25.9%) when compared with Caucasians or Japanese, but it has similar heterozygosity in Koreans and Chinese.

Analysis of the *Xba*I polymorphism by Southern blot is known to be very informative in Caucasians, Chinese, and Japanese, giving a heterozygosity rate of 43%–49% (Wion et al. 1986; Chan et al. 1988; Suehiro et al. 1988; Salle et al. 1990). However, analysis of the *Xba*I polymorphism by PCR is not reliable, because the homologous non-factor VIII region on the X chromosome, which, for the most part, does not contain the *Xba*I restriction site, is also coamplified (Kogan et al. 1987). This problem of coamplification has led us to abandon analysis of the *Xba*I polymorphism by PCR in the carrier detection and prenatal diagnosis of the hemophilia A.

Southern blot analysis of *Taq*I/St14 (DXS52) VNTR reveals a polymorphic system comprising at least ten allelic fragments, with an ethnic variation in allele frequencies. This *Taq*I/St14 RFLP gave a heterozygosity rate of over 80% in Caucasians (Oberle et al. 1985; Janco et al. 1986; Janco et al. 1987), 76% in Japanese (Suehiro et al. 1988), and 85% in Chinese (Chan et al. 1988).

Recently, PCR analysis of St14 VNTR has been introduced for the rapid analysis and discrimination of closely sized alleles (Richards et al. 1991). However, there have been few reports on this subject to date. Richards et al. (1991) have found 14 alleles in Caucasians, ranging from 700 bp to 3000 bp. We revealed 15 alleles in the Korean population, ranging from 700 bp to 2890 bp. The expected heterozygosity rate of 78% in Koreans was comparable to that found in Japanese and Chinese by Southern blot analysis (Chan et al. 1988; Suehiro et al. 1988).

Compared with Caucasians, there was a markedly higher occurrence of low molecular weight alleles in Koreans. The most frequent allele in Koreans was 700 bp in size, while it was 1690 bp in size in Caucasians (Richards et al. 1991). The allele frequencies found in Koreans were similar to those found in the Chinese population (Wang et al. 1995), in which the most frequent allele was 700 bp.

St14 VNTR is a highly informative marker in the molecular diagnosis of hemophilia A, but it carries a risk of about 4% recombination leading to misdiagnosis (Gitschier et al. 1991). In the course of this study, we experienced one case of misdiagnosis using St14 VNTR analysis. This type of misdiagnosis, due to the use of extragenic markers, can be prevented by the use of other intragenic markers, such as intron 13/22 dinucleotide repeat polymorphisms.

The combination of the *Bcl*I polymorphism and *Taq*I/St14 determined by Southern blot analysis in the Italian population showed that 91% of obligate carriers were heterozygous for one or both markers (Pecorara et al. 1987). Our study disclosed that the combined use of these two polymorphic markers by PCR gave a heterozygosity rate of 81.9% in the Korean population.

Besides the two polymorphisms we have analyzed in this study, we have recently started to analyze intron 13/22 dinucleotide repeat polymorphisms and factor VIII gene inversion in a Korean population. Our preliminary study showed that intron 13/22 dinucleotide repeat polymorphisms had an observed heterozygosity of about 80%, and that factor VIII gene inversion was found in about 40% of patients with severe hemophilia A (unpublished results). These preliminary data are consistent with other reports, in the Caucasian population (Lalloz et al. 1991; Lakich et al. 1993; Windsor et al. 1994). Therefore, the useful markers for carrier detection and prenatal diagnosis of hemophilia A in the Korean population are thought to be the *Bcl*I/intron 18 polymorphism, intron 13/22 dinucleotide repeat polymorphisms, factor VIII gene inversion, and St14 VNTR.

Some investigators have reported factor VIII levels in cord blood during the midtrimester (Mibashan et al. 1979; Mibashan et al. 1980; Forestier et al. 1988). Forestier et al. (1988) reported normal factor VIII activity of 39 ± 12% between 18 and 22 weeks of gestation. In our subjects, normal factor VIII activity ranged from 21% to 125% between 18 and 24 weeks of gestation. Mibashan et al. (1980) reported abnormal factor VIIIC levels of 1 to 3 U/dl, in ten fetuses from families with severe hemophilia A between 17 and 22 weeks of gestation, whereas normal values in that gestational period were 31 to 81 U/dl (mean, 44 U/dl). In this study, we found that values for factor VIII activity in affected fetuses were less than 5% in cord blood. It is recommended that centers involved in the prenatal diagnosis of hemophilia A have their own data on factor VIII levels in fetuses.

In conclusion, our study has shown that the PCR-based analysis of the *Bcl*I/intron 18 and St14 VNTR polymorphisms is useful in the carrier detection and prenatal diagnosis of hemophilia A in the Korean population.

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