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Separation of intron 22 inversion type 1 and 2 of hemophilia A by modified inverse-shifting polymerase chain reaction and capillary gel electrophoresis

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ABSTRACT

An inverse-shifting polymerase chain reaction (IS-PCR) combined with short-end capillary gel electrophoresis (CGE) was developed for genotyping of intron 22 inversion Type 1 (Inv22-1) and Type 2 (Inv22-2) of hemophilia A (HA). Severe HA cases are affected by intron 22 inversion around 45–50%. Inv22-1 has higher frequency than Inv22-2. The aim of this study is to distinguish them by genotyping. In order to improve Inv22 genotyping efficiency, five primers were designed and applied to differentiate the wild type, Inv22-1, Inv22-2 and carrier. Three amplicons of 405, 457 and 512 bp were recognized for wild type; 333, 457 and 584 bp for Inv22-1; 385, 405 and 584 bp for Inv22-2. The Inv22-1 carrier has 5 amplicons including 333, 405, 457, 512, 584 bp and Inv22-2 carrier is differentiated by 385, 405, 457, 512 and 584 bp. The amplicons between Inv22-1 and Inv22-2 carriers are only different in 333 bp for Inv22-1 carrier and 385 bp for Inv22-2 carrier. Capillary gel electrophoresis (CGE) was used for separation within 5 min. The separation voltage was set at 8 kV (cathode at detector), and the temperature was kept at 25 °C. The sieving matrix was 89 mM Tris, 89 mM boric acid, 2 mM EDTA containing 0.4% (w/v) HPMC and 1 μM of YO-PRO®-1 Iodide. Total of 50 HA patients (including 35 non-Inv22, 14 Inv22-1, and one Inv22-2 patients) and 7 HA carriers were diagnosed in the application. Seven random samples (5 patients and 2 carriers) were subjected to comparison and gave identical results of DNA sequencing and this modified IS-PCR.

1. Introduction

Hemophilia A (HA) is a congenital X-linked recessive bleeding disorder caused by defects of the gene located at the distal end of the long arm of X chromosome (Xq28), which encodes coagulation factor VIII (*F8*) [1]. In 1984, the HA gene was first cloned and elucidated [2,3]. The incidence of this disease has been estimated to be around 1 in 5000–10,000 male live births and rarely in females (females are usually carriers). Until now, more than 2107 disease-causing defects of *F8* gene have been reported, including gene inversion, point mutations (missense, nonsense, splice site and frame shift) and deletion [4,5]. The most common defects of *F8* gene are the intron 1 inversion (Inv1) and intron 22 inversion (Inv22), which account for 2% [6,7] and 45–50% [8–10] of severe HA, respectively. Inv22 event is a result of intra-chromosomal homologous recombination between the int22h-1 region within the *F8* locus and extragenic int22h-2 (intron 22 inversion type 2, Inv22-2) or int22h-3 (intron 22 inversion type 1, Inv22-1) regions, located approximately 500 kb and 600 kb away from *F8* gene, respectively [11,12]. Hence, intrachromosomal homologous recombination between int22h-1 and either int22h-2 or int22h-3 causes separation of exons 1-22 from exons 23-26 and puts them in orientations opposite to int22h-1 (Fig. 1). Among affected patients with Inv22, 87% have the inversion between int22h-1 and int22h-3 (Inv22-1) and 13% between int22h-1 and int22h-2 (Inv22h-2) [13]. Inv22h-1 was reported to have higher frequency than Inv22h-2 [14,15,19]. Yoshioka et al. reported an interesting phenomenon that all of the detected HA patients were Inv22-1, although these results did not have statistically significant differences [16]. As a result, Inv22 genotyping is very helpful and important for guiding the direction of molecular studies and for understanding

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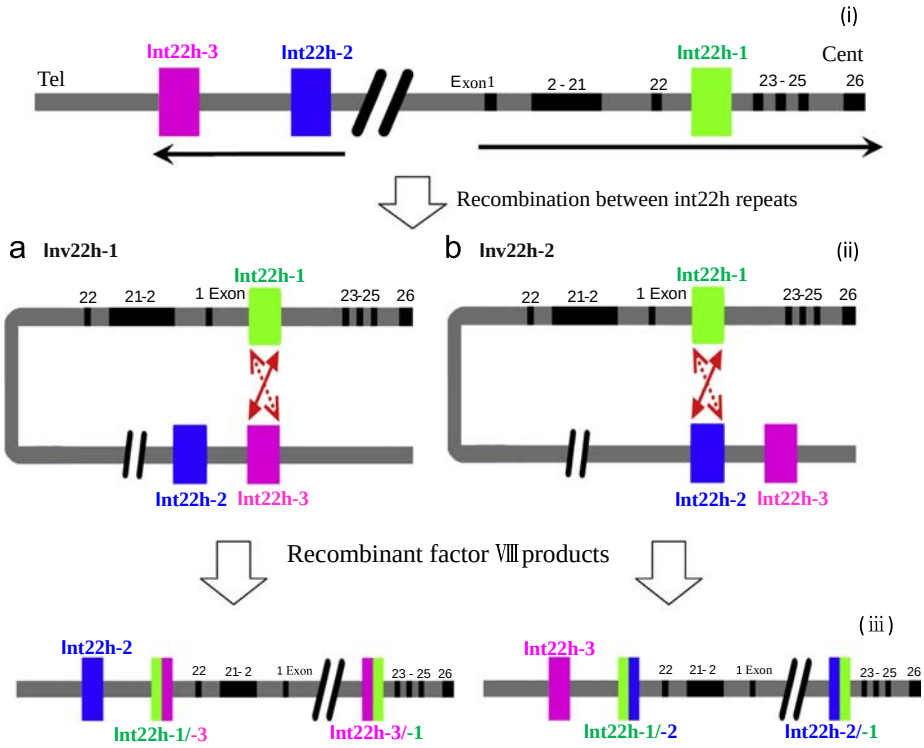


Fig. 1. Schematic representation of (i) chromosomal localization, (ii) the *F8* gene structure and (iii) mechanism of intron 22 inversions (Inv22). The 3 intron 22 homologous regions int22h-1, int22h-2 and int22h-3 are represented as green, yellow and red squares, respectively. Arrows indicate the direction of transcription of *F8* gene. Tel indicates telomere; Cent indicates centromere. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

about intron 22–related rearrangements, including Inv22-1, Inv22-2 and intron 22 deletions and duplications.

In previous researches, different approaches for identifying defects of *F8* gene have been used for detection of more than 2000 mutations within HA [4,17]. Over the last decades, a method using long-distance polymerase chain reaction (LD-PCR) [18] was used to detect Inv22 of *F8* gene in HA. In 2008, a new technique, inverse-shifting polymerase chain reaction (IS-PCR), has been reported for detection of Inv22 in severe HA patients [19]. This strategy is unlike normal PCR system and needs to use restriction endounclease to digest the double-stranded DNA. The restriction enzyme of *BclI* can recognize the sequences of 5′-T↓GATCA-3′ and generate fragments having 5′- cohesive termini, which is followed by self-ligation to form a circular DNA. LD PCR could be used for diagnosis of Inv22 of F8 gene, but not for type 1 and 2. In this method, we modified the IS-PCR, designed two more primers, and optimized the PCR conditions. Obviously, modified IS-PCR analysis offers a convenient method for genotyping of Inv22 (including Inv22-1, Inv22-2, Del22 and Dup22) and carriers in HA patients and their families [14,16,19].

Capillary gel electrophoresis (CGE) is a preferred instrumental technique for genotyping as it offers high efficiency, resolution, speed and qualitative ability [20–22]. In recent years, CGE has been viewed as a powerful electromigration method for screening genetic mutations, diagnosing genetic disorders and single-nucleotide polymorphism in DNA research. It is a rapid and inexpensive method compared to other techniques [23,24]. In CGE, the sample is usually introduced into the capillary from the inlet and the distance between inlet and detector was the longest length used for separation of DNA fragments [25–28]. As described by previous researches [22,29–33], in comparison with long-end injection modes, the short-end capillary in which the sample is injected from the outlet can provide a notably fast analysis by employing a shorter separation distance. Therefore, short-end CGE is more suitable for fast separation. Until now, no CGE method has been developed for high-speed detection of Inv22 Type 1 and Type 2 in HA.

2. Experimental section

2.1. Chemicals and reagents

Tris (hydroxymethyl) aminomethane (Tris)-borate-EDTA (TBE) buffer (5 ×) was (obtained from Protech Technology Enterprise) diluted to the necessary concentration with double-deionized water before use. The double-deionized water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). Intercalating dye, YO-PRO®-1 Iodide, was purchased from Molecular Probes (Invitrogen detection technologies, Eugene, OR, USA). The commercial synthetic PAGE-grade primers listed in Table 1 were purchased from Genomics BioSci & Tech (New Taipei City, Taiwan). T4 DNA ligase and *BclI* enzyme were purchased from Takara Biotechnology (Japan) and New England Biolabs (Beverly, MA, USA), respectively. Hydroxyethylcellulose (HEC) (molecular weight about 80,000) (Fluka, Seelze, Germany), hydroxypropyl cellulose (HPC, molecular weight about 80,000) (Aldrich), hydroxypropyl-methylcellulose (HPMC) (molecular weight about 90,000) (Aldrich) and polyethylene oxide (PEO) (molecular weight about 80,000) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Genomic DNA extraction

The genomic DNA was isolated according to the protocols followed in previous researches [34–36]. Five milliliters of peripheral blood was collected by using ethylene diamine tetraacetic acid (EDTA) tubes. Genomic DNA in the whole blood was purified by using phenol and chloroform and was precipitated by ethanol. After DNA extraction, the amount and purity of DNA samples were measured by Nanodrop-2000 spectrophotometer (Nano-drop

Technologies, Wilmington, DE). The absorbance ratio of 260 nm/280 nm was determined to be around 1.8–2.0. The crude DNA was diluted to 1 μg/μL by water before digestion. This research was approved by the institutional review board (IRB) of Chung-Ho Memorial Hospital, Kaohsiung Medical University.

Table 1
Sequences of PCR primers.

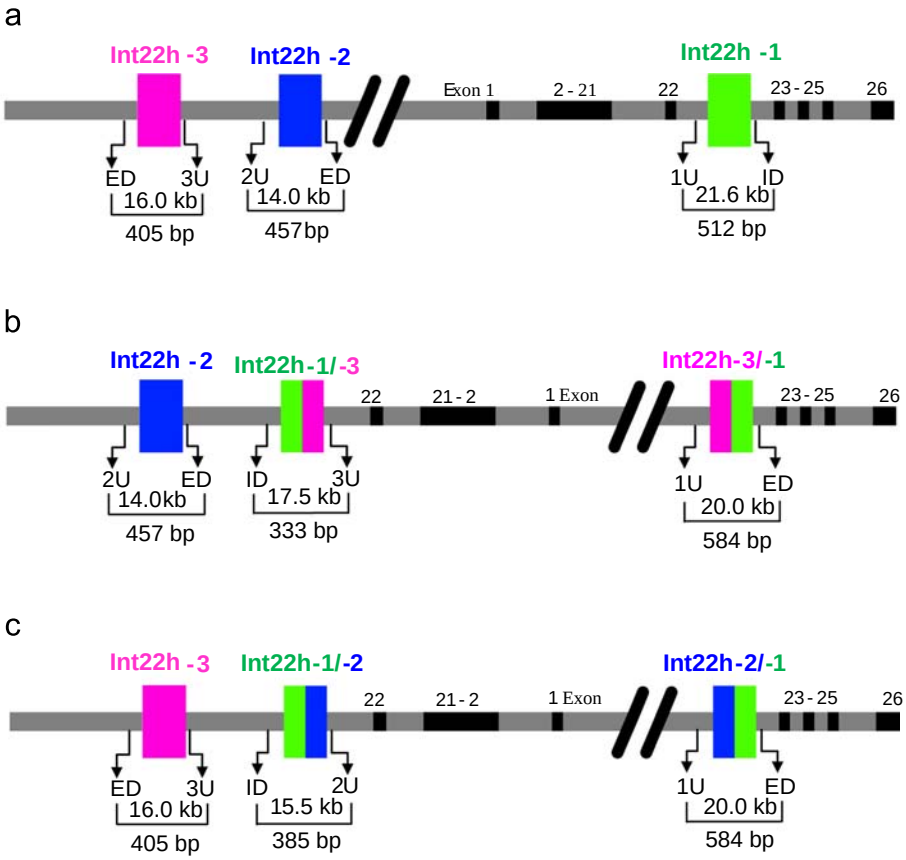
Gene	Primer	Sequence (5′→3′)	*BclI* site*	Primer length (bp)
Intron 22	22h-1U	AACTCCCTTCCTTGTCAGCA	485	20
	22h-2U	ACGTGTCTTTTGGAGAAGTC	358	20
	22h-3U	CTCACATTGTGTTCTTGTAGTC	306	22
	IPCR-ID	ACATACGGTTTAGTCACAAGT	27	21
	IPCR-ED	TCCAGTCACTTAGGCTCAG	99	19
DMD	E45-F	AACGACTGCATGTGGTAGCACACTG	–	25
	E45-R	TTGTACCTCTTTGGCTCAAG	–	20

* DNA distance (bp) from the 5′ end of the primer to the *BclI* restriction site. This DNA distance indicates the impact of usage of this primer on the modified IS-PCR product.

2.3. Modified IS-PCR strategy and protocol

In modified IS-PCR method, five primers were used for producing different fragments indicating the genotypes of Inv22 (Fig. 2). As shown in Fig. 2A, three amplicons of 405, 457 and 512 bp were recognized for wild type (non-Inv22); 333, 457 and 584 bp for Inv22-1(as shown in Fig. 2B); 385, 405 and 584 bp for Inv22-2 (as shown in Fig. 2C). The Inv22-1 carrier has 5 amplicons including 333, 405, 457, 512, 584 bp and Inv22-2 carrier is differentiated by 385, 405, 457, 512 and 584 bp. The amplicons between Inv22-1 and Inv22-2 carriers are only different in 333 bp for Inv22-1 carrier and 385 bp for Inv22-2 carrier. In this way, different Inv22 genotypes can be recognized, according to the 6 amplification products amplified by the designed 5 primers.

Modified IS-PCR was performed according to the guidelines described in previous studies [18,36,37]. We modified this amplification, designed our primers and optimized the PCR conditions. First, the genomic DNA was digested by using the *BclI* enzyme (New England Biolabs, Beverly, MA, USA) at 50 °C for 2 h. The total of 50 μL reaction volume contained 15U of the digestion enzyme and 5 μg genomic DNA in 1× reaction buffer. And then the digested DNA fragments were purified by 3 mM sodium chloride (0.11 volumes) and 2 volumes of ethanol, washed by 75% alcohol twice and were resuspended in 10 μL of sterile water.



Intron 22 Primer	22h-1U	22h-2U	22h-3U
IPCR-ID (bp)	512	385	333
IPCR-ED (bp)	584	457	405

Fig. 2. Schematic of IS-PCR for genotyping intron 22-related rearrangements. (a) non-Inv22 (WT); (b) intron 22 inversion type 1 (Inv22-1); (c) intron 22 inversion type 2 (Inv22-2).

Self-circularized ligation was followed by using 100 μL reaction volumes with 3 U of T4 DNA ligase (Takara Biotechnology, Japan) in 1 × ligation buffer provided by the manufacturer and carried out at 15 °C for 12 h. The self-circularized fragments were then isolated by 3 mM sodium chloride (0.11 volumes) and 2 volumes of ethanol and then washed by 75% alcohol twice and resuspended in 10 μL of sterile water.

2.4. Multiplex PCR procedures

In this experiment, a multiplex PCR in a single tube was utilized to achieve the diagnosis of intron 22 genotypes. The PAGE-grade primers used are as shown in Table 1. The Duchenne muscular dystrophy gene (DMD) was used as an extra internal standard (IS), according to a previously used method [26,27]. Genomic DNA was used as a template for amplification of DMD gene that was carried out in another PCR tube. The final reaction volume was 25 μL containing 200 ng of purified circularized DNA, 200 μM deoxy nucleoside triphosphate (dNTP), 1 unit of Takara rTaq and the proper concentration of each primer as previously described [19,29]. PCR reactions were performed in a Tprofessional thermocycler (Biometra Germany). The first amplification was performed with an initial denaturing step at 95 °C for 10 min, followed by thirty cycles of denaturing at 95 °C for 30 s, annealing at 57 °C for

Table 2
Comparison of time consuming between these two methods.

	IS-PCR with AGE	This modified IS-PCR with short-end CGE
Digestion reaction	4 h	2 h
Ligation reaction	Overnight (16–18 h)	12 h
Purification of digested and circularized DNA	Phenol-Chloroform (about 2.5 h)	Salting-out (0.5 h)
PCR reaction	2 h	2 h
Analysis time	50 min	5 min
Total	27.33 h	16.58 h

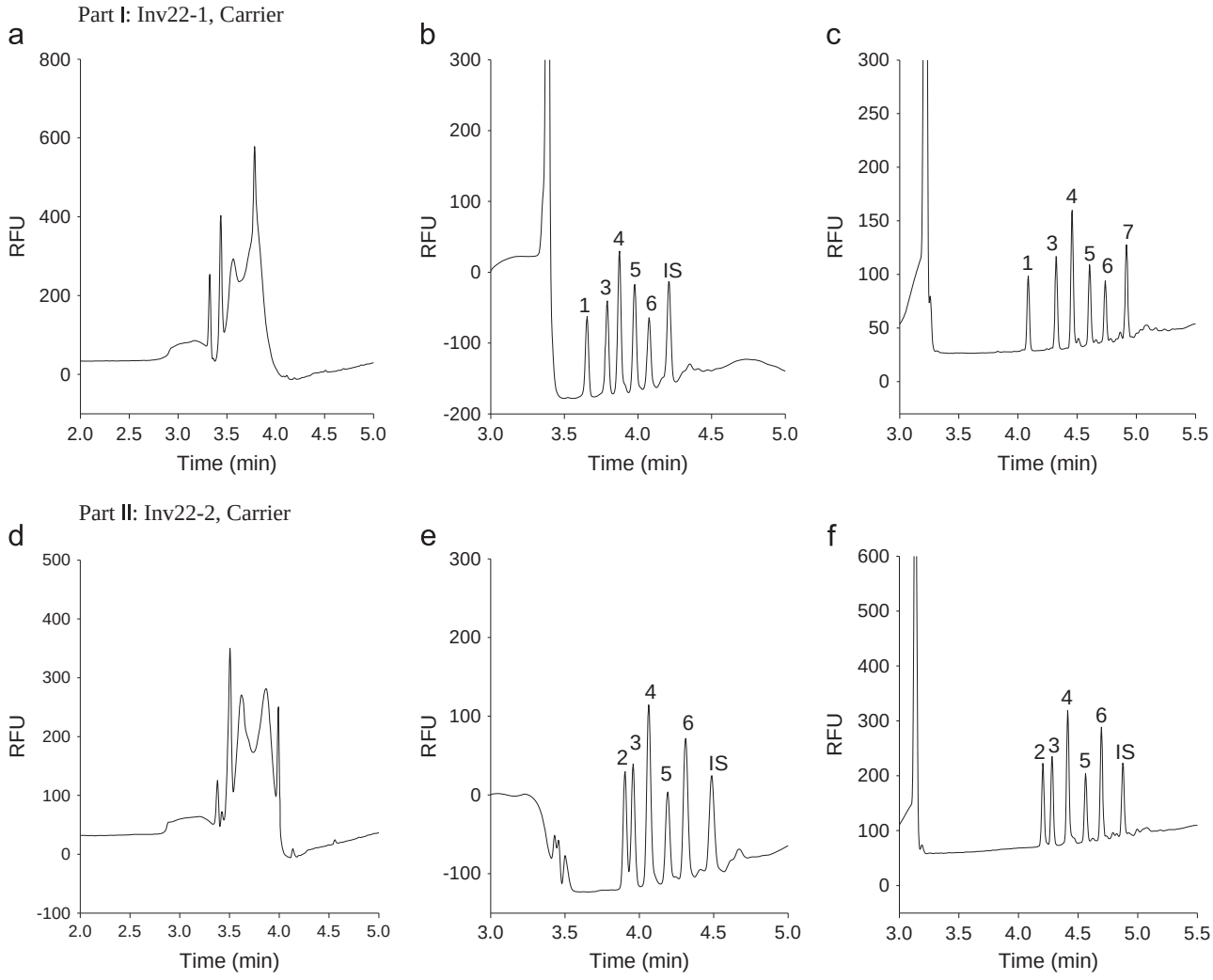


Fig. 3. Effects of different kinds of polymer solutions (HPC, HEC, HPMC) on the separation of Inv22 -1 (part I) and Inv22-2 (part II)—Carrier. ((a) and (d)) 0.4% HPC, ((b) and (e)) 0.4% HEC, ((c) and (f)) 0.4% HPMC (w/v). CE condition: applied voltage, 8 kV (detector at cathode side); DB-17 capillary, (effective length) × 100 μm I.D.; injection, 10 kV, 20 s; temperature, 25 °C. Peaks identified: 1. 333 bp; 2. 385 bp; 3. 405 bp; 4. 457 bp; 5. 512 bp; 6. 584 bp; IS. 759 bp.

1 min, extension at 72 °C for 90 s and then a final extension step at 72 °C for 10 min. Prior to CGE analysis, the PCR products and IS are diluted five-fold and ten-fold with deionized water.

2.5. Short-end CGE system

The separation of amplicons of int-22 inversions was performed on a P/ACE MDQ system (Beckman, Fullerton, CA, USA) equipped with a laser-induced fluorescence detector and a liquid-cooling device. Detection of fluorescence was at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. Separations were performed in a coated DB-17 capillary (Agilent Technologies) with 100 μm internal diameter and 10.2 cm effective length (detection window was made at 10.2 cm from the outlet to detection window of the capillary). Prior to first use, the capillary was pre-conditioned by washing for 10 min with MeOH and for another 10 min with deionized water. After filling the capillary with sieving matrix, samples were injected by electrokinetic injection at 10 kV for 20 s. The separation voltage was set at 8 kV (cathode at detector), and the temperature was kept at 25 °C. The sieving matrix was 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) (Amresco, Solon, OH, USA) containing 0.4% (w/v) HPMC and 1 μM of YO-PRO®-1 Iodide.

3. Results and discussion

In this research, we used a modified IS-PCR assay combined with short-end CGE method for detection of Inv22 genotypes. The modified IS-PCR included genomic DNA digestion by the restriction endonuclease (*BClI*), followed by self-ligation to produce *BClI* circules and amplification by multiplex PCR. The modified IS-PCR is based on the two reverse primers (IPCR-ID and IPCR-ED) and three forward primers (22h-1U, 22h-2U and 22h-3U) designed to diagnose the genotypes of intron 22–related rearrangements. However, the major limitation of this method is restriction endonuclease (*BclI*) properties that only cuts specific recognition nucleotide sequences that caused a problem with other point mutation detection of *F8* in HA patients. Moreover, compared to the traditional IS-PCR combined with agarose gel electrophoresis (AGE), this modified IS-PCR with short-end CGE could save 10 h (as shown in Table 2). For optimization of short-end CGE system to obtain the better separation efficiency, the different types of

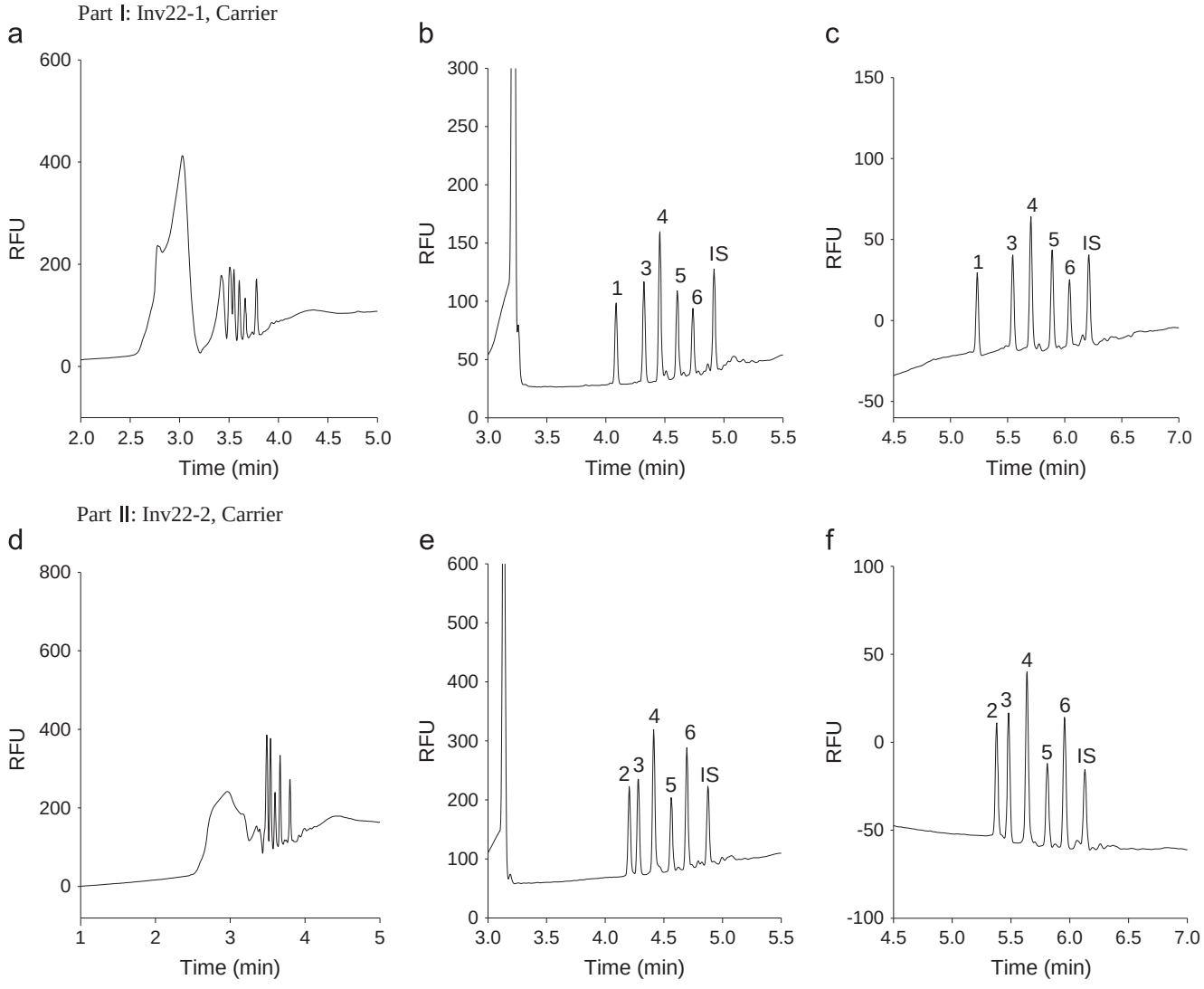


Fig. 4. Effect of polymer concentration on the separation of Inv22-1 (part I) and Inv22-2 (part II)—Carrier. ((a) and (d)) 0.2%, ((b) and (e)) 0.4%, ((c) and (f)) 0.8% HPMC (w/v) in 1 × TBE. Peaks identified: 1, 333 bp; 2, 385 bp; 3, 405 bp; 4, 457 bp; 5, 512 bp; 6, 584 bp; IS, 759 bp. CE conditions were the same as in Fig. 3.

symptomatic carriers of HA were used to evaluate the separation conditions. Several parameters that affected the assay method were investigated, including polymer types, polymer concentrations and separation voltages.

3.1. Polymer selection and optimization

Hydrophilic polymer solutions are becoming more and more popular for separation of DNA fragments in CGE. These include PEO and cellulose derivatives (such as HPC, HEC and HPMC). The polymer solutions have a number of advantages, such as low toxicity, flexibility, availability, unique physical properties and others. As a result, the polymer solutions have the advantages of high sieving ability, low viscosity and easy to prepare as separation media [38,39]. The influence of different polymer types (HPC, HEC and HPMC) was investigated. Fig. 3 shows the effect of different types of polymer solutions, where the PCR products of Inv22-1 and Inv22-2 carriers were individually evaluated. By using 0.4% HPC polymer solution, the Inv22-1 and Inv22-2 carriers could not be separated well (Fig. 3a and d). When the 0.4% HEC was used (Fig. 3e), the peaks of 2 and 3 in the sample of Inv22-2 carrier became apparently broad without base-line separation. Based on comparison of the results (Fig. 3f), finally, 0.4% HPMC was chosen for further investigation. The polymers of HPMC and PEO were also evaluated for separation of Inv22-1 and Inv22-2 carriers. These two polymer solutions had similar resolution, peak shape and low viscosity, but the migration when using 0.4% HPMC was faster than when using 0.4% PEO (about 30 s, data not shown). Therefore, 0.4% PEO was not selected as the separation solution.

Effects of different HPMC concentrations in the sieving matrix (0.2, 0.4 and 0.8% w/v) on genotyping of Inv22-1 and Inv22-2 carriers were investigated. 0.2% HPMC failed to resolve the seven peaks (Fig. 4a) because of the low sieving ability for separation of DNA fragments. When the HPMC concentration was raised to 0.8%, we found the peaks could be well separated. However, higher concentrations resulted in higher viscosity and slower migration of DNA fragments. Considering the separation efficiency, buffer viscosity and analysis time, 0.4% HPMC was selected for the separation.

3.2. Effect of separation voltage

Separation voltage is one of the most important parameters in optimization of CGE because it can affect both the electroosmotic flow of the system and the migration times for each analyte [40]. Therefore, the effect of separation voltage on this method was evaluated. Several separation voltages were investigated (4, 6, 8 and 10 kV). The Inv22-2 of symptomatic carrier of HA was not well separated at 10 kV. Considering the speed, current and resolution, 8 kV was chosen for the experiment.

3.3. Validation of short-end capillary in CGE

In order to verify the performance of the short-end CGE separation, reproducibility and relative migration time (RMT) with internal standard (IS) under the optimum conditions described above were studied. Ten independent runs were carried out for investigation of reproducibility of the migration time by short-end CGE. Table 3 summarizes the migration times of seven peaks and relative standard deviations (%RSD) of migration times in this study. The RSD values of migration times were less than 2.48% without addition of IS. When an IS was added to calibrate the migration time of the seven peaks, RSD values of relative migration times (RMT) of the seven peaks were less than 0.33%, much smaller than without addition of IS (Table 4). The relative migration time was calculated from t_m/t_{IS}. The results indicated that the

Table 3
Reproducibility in migration time (MT) for genotypes of Inv22 ($n=10$).

Peak[a]	Inv22-1-carrier		Inv22-2-carrier		Wild type		Inv22-1		Inv22-2	
	MT (min)	%RSD[b]	MT (min)	%RSD	MT (min)	%RSD	MT (min)	%RSD	MT (min)	%RSD
1	3.78 ± 0.05	1.31	–	–	–	–	3.96 ± 0.08	2.01	–	–
2	–	–	3.83 ± 0.06	1.56	–	–	–	–	4.14 ± 0.10	2.38
3	3.99 ± 0.05	1.24	3.90 ± 0.06	1.45	4.21 ± 0.10	2.45	–	–	4.21 ± 0.10	2.43
4	4.10 ± 0.05	1.20	4.01 ± 0.06	1.48	4.33 ± 0.10	2.39	4.30 ± 0.09	2.02	–	–
5	4.24 ± 0.05	1.21	4.14 ± 0.06	1.56	4.47 ± 0.10	2.33	–	–	–	–
6	4.35 ± 0.05	1.15	4.25 ± 0.07	1.59	–	–	4.57 ± 0.10	2.08	4.60 ± 0.11	2.47
IS	4.50 ± 0.05	1.10	4.40 ± 0.07	1.66	4.75 ± 0.11	2.38	4.74 ± 0.10	2.12	4.75 ± 0.12	2.48

[a] Peaks identified: 1. 333 bp; 2. 385 bp; 3. 405 bp; 4. 457 bp; 5. 512 bp; 6. 584 bp; IS. 759 bp.
[b] RSD: relative standard deviation.

Table 4
Reproducibility in the relative migration time (RMT) for genotypes of Inv22 ($n=10$).

Peak[a]	Inv22-1-carrier		Inv22-2-carrier		Wild type		Inv22-1		Inv22-2	
	RMT[b] (min)	%RSD[c]	RMT (min)	%RSD	RMT (min)	%RSD	RMT (min)	%RSD	RMT (min)	%RSD
1	0.841 ± 0.003	0.33	–	–	–	–	0.834 ± 0.002	0.27	–	–
2	–	–	0.871 ± 0.001	0.13	–	–	–	–	0.869 ± 0.002	0.18
3	0.886 ± 0.002	0.18	0.886 ± 0.002	0.20	0.886 ± 0.001	0.12	–	–	0.884 ± 0.001	0.15
4	0.910 ± 0.001	0.16	0.911 ± 0.002	0.21	0.911 ± 0.001	0.07	0.906 ± 0.002	0.17	–	–
5	0.941 ± 0.002	0.16	0.940 ± 0.001	0.12	0.940 ± 0.001	0.13	–	–	–	–
6	0.966 ± 0.001	0.10	0.966 ± 0.001	0.13	–	–	0.964 ± 0.001	0.09	0.966 ± 0.001	0.11
IS	1.000	–	1.000	–	1.000	–	1.000	–	1.000	–

[a] Peaks identified: 1. 333 bp; 2. 385 bp; 3. 405 bp; 4. 457 bp; 5. 512 bp; 6. 584 bp; IS. 759 bp.
[b] RMT: relative migration time calculated from t_m/t_{IS}.
[c] RSD: relative standard deviation.

short-end CGE method could provide good reproducibility and excellent precision.

3.4. Effect of long-end injection

The DNA samples of Inv22-1 and Inv22-2 carriers were also analyzed by long-end injection. It needed 15 min. For short-end injection mode, 5 min was sufficient for separation.

Table 5
Results of intron 22-related rearrangements of *F8* gene by modified IS-PCR in HA patients and carriers.

Genotypes of intron 22 related-rearrangements	Count
HA patients	
Non-intron 22 inversion	35
Intron 22 inversion type 1 (Inv22-1)	14
Intron 22 inversion type 2 (Inv22-2)	1
HA carriers	
Non-intron 22 inversion	3
Intron 22 inversion type 1 (Inv22-1)	3
Intron 22 inversion type 2 (Inv22-2)	1
Total	57

3.5. Applications

From the above results, the optimal separation conditions of the short-end CGE were set as 1 × TBE buffer solution containing 0.4% HPMC and separation voltage of 8 kV. Under optimum separation conditions, this method was used to detect Inv22 genotypes in *F8* genes of 50 HA patients (35 Inv22-negative, 14 Inv22-1, and 1 Inv22-2) and 7 carriers. Many studies have reported the prevalence of Inv22 of *F8* gene in different countries is quite widespread, in a range of 17.6–46.8% [41,42]. The diagnostic results of intron 22-related rearrangements of *F8* gene were shown in Table 5. Fifty HA patients and 7 HA carriers were examined. Among them, thirty-five HA patients and three HA carriers were non-intron 22 inversion, fourteen HA patients and three HA carriers were Inv22-1, one HA patients and one HA carriers were Inv22-2. In this study, we found 14 (28%) Inv22-1 and 1 (2%) Inv22-2 among 50 HA patients, indicating the frequency of Inv22-1 is higher than Inv22-2. Overall, the prevalence of Inv22 was detected in 30% of the 50 HA patients examined with this proposed method, which is close to the 35% referred from previous study [40]. Fig. 5 shows, the electropherograms obtained from analysis of the WT, Inv22-1, Inv22-2, WT/Inv22-1 (carrier-1) and WT/Inv22-2 (carrier-2) genotypes using the short-end CGE combined with IS-PCR method. Seven random samples (three Inv22-positive patients, two Inv22-negative patients, and two carriers)

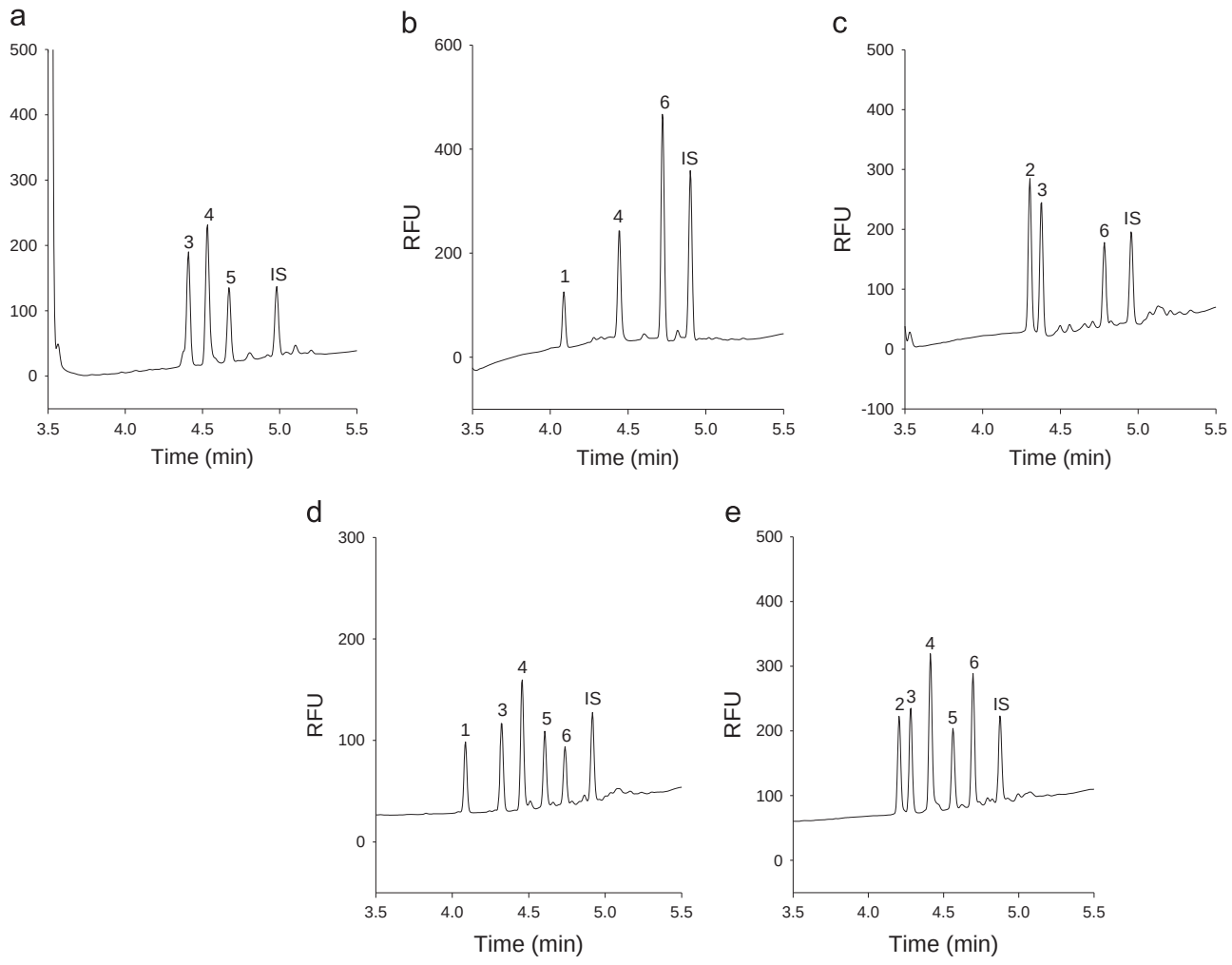


Fig. 5. Electropherograms of different genotypes from HA patients by short-end capillary in CGE. (a) Wild type (WT; non-Inv22) (peak 3, 4, 5, IS), (b) Inv22-1 (peak 1, 4, 6, IS), (c) Inv22-2 (peak 2, 3, 6, IS), (d) WT/ Inv22-1 (carrier) (peak 1, 3, 4, 5, 6, IS), (e) WT/ Inv22-2 (carrier) (peak 2, 3, 4, 5, 6, IS). Peaks identified: 1, 333 bp; 2, 385 bp; 3, 405 bp; 4, 457 bp; 5, 512 bp; 6, 584 bp; IS, 759 bp. Other CE conditions were the same as in Fig. 3.

were subjected to comparison by DNA sequencing and this modified IS-PCR. The results were coincident. However, Del22 and Dup22 were not found in our samples of HA patients, but modified IS-PCR still has great potential to discrimination of int22h rearrangements, including Del22 and Dup22 [14,16,19].

4. Conclusions

This study was the first to use high-speed CGE method for detection of Inv22 and distinguishing them into Type 1 and Type 2 of the Inv22, in the *F8* gene. In this research, the proposed method is demonstrated to be an ideal diagnostic tool for rapid genotyping of Inv22 in *F8* gene by using the modified IS-PCR system combined with short-end CGE. Our genotyping results of 3 Inv22-positive patients, 2 Inv22-negative patients, and 2 carriers were coincident with DNA sequencing, and showed the accuracy of this modified IS-PCR CGE method. In this way, CGE with short-end capillary is a simple, reproducible and robust method. Additionally, the short-end CGE method effectively reduces the total analysis time compared to previous methods that used the long-end injection. The present study only used the salting-out procedure to extract circularized DNA, while IS-PCR used phenol-chloroform to extract it. This method is a rapid, feasible and useful technique for identification of Inv22 genotypes and carriers in clinical analysis.

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