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Profiling of Mutations in the *F8* and *F9*, Causative Genes of Hemophilia A and Hemophilia B

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1. Introduction

Hemophilia, a common congenital coagulation disorder, is classified as hemophilia A (HA) and hemophilia B (HB), which result from a deficiency or dysfunction of coagulation factor VIII (FVIII) and factor IX (FIX), respectively. HA is known to be caused by heterogeneous mutations of the FVIII gene (*F8*), such as inversions, substitutions, deletions, insertions, etc. *F8* (NM_000132.3) is located on the long arm of the Xq28 region of the X chromosome. *F8* is extremely large (186 kb) and consists of 26 exons (Graw et al., 2005). The transcript of *F8* is approximately 9010 bp and comprises a short 5′-untranslated region (5′-UTR; 150 bp), an open reading frame (ORF) plus stop codon (7056 bp), and a long 3′-UTR (1806 bp). The protein product of *F8* is a cofactor of FIX, without enzyme activity. The ORF encodes a signal peptide with 19 amino acids at its N-terminus, which leads to the passage of FVIII through hepatocytes to blood vessels. The matured FVIII protein contains 2332 amino acids and a glycoprotein of approximately 250 kDa, and circulates as an inactive pro-cofactor.

FVIII is a multi-domain protein composed of A1-A2-B-A3-C1-C2, named from the N-terminus. FVIII synthesized in hepatocytes is secreted into the circulation and readily assembled with von Willebrand factor (vWF), which is generated and secreted by endothelial cells. Besides vWF, FVIII protein can also interact with diverse proteins such as thrombin and FX. These interactions are important for effective hemostasis. However, *F8* mutations can lead to the production of truncated proteins, which lead to disruption of FVIII function and suppress normal protein interaction with proteins involved in the coagulation cascade (Bowen, 2002). This inappropriate reaction causes bleeding tendency.

F8 mutations can occur at diverse sites in a variety of types, such as structural variation (inversions of intron 22 or intron 1) and sequence variation (insertion, deletion, and substitution). The latter variation leads to nonsense, missense, and frameshift mutations. Recently, more than 1,200 types of *F8* mutations were reported in the HAMSTeRS (Hemophilia A Mutation, Structure, Test and Resource Site) database (http://hadb.org.uk).

The *F9* gene (NM_000133.3) is also located on the X chromosome at Xq27.1–q27.2. In contrst to *F8*, the size of *F9* gene is approximately 34 kb with only eight exons and the size of the transcript mRNA is 2803 bp. The *F9* gene encodes the FIX protein, one of the vitamin

K-dependent coagulation factors in humans. FIX is synthesized in the liver as 461 amino acid residues, including 46 signal peptides at its N-terminus. It circulates in the blood as a single-chain glycoprotein of inactive zymogen (Yoshitake et al., 1985). When coagulation is initiated, FIX is converted to an active form (FIXa) by proteolytic cleavage, resulting in an N-terminal light chain and a C-terminal heavy chain held together by one or more disulfide bonds (Di Scipio et al., 1978; Lindquist et al., 1978). The role of FIXa in the blood coagulation cascade is to activate factor X through interactions with calcium ions, membrane phospholipids, and FVIII.

More than 1,000 mutations have been reported for *F9* to date (http://hadb.org.uk). The data archived in the locus-specific mutation database for *F9* (http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html) describe the genotype-phenotype correlations. Although the mutations are scattered over the entire structure of the *F9* gene, the distribution of mutation types shows that missense/nonsense mutations are the most common, accounting for ~64% of mutations, followed by frameshift mutations (~17%). More than 90% of mutations are point mutations that can be detected by direct sequencing analyses (Mahajan et al., 2007). The rest (<10%) consist of large exon deletion mutations or complex rearrangements. Unlike in HA, mutations with large inversion rearrangement are rare in HB.

2. Profiling of the *F8* mutations

The profiling of *F8* mutations is important for a precise diagnosis of HA, understanding of genotype-phenotype correlation, carrier detection, prenatal diagnosis, and predicting inhibitor development. As there are various types of mutations, we propose a strategy for profiling *F8* mutations as follows (Figure 1)

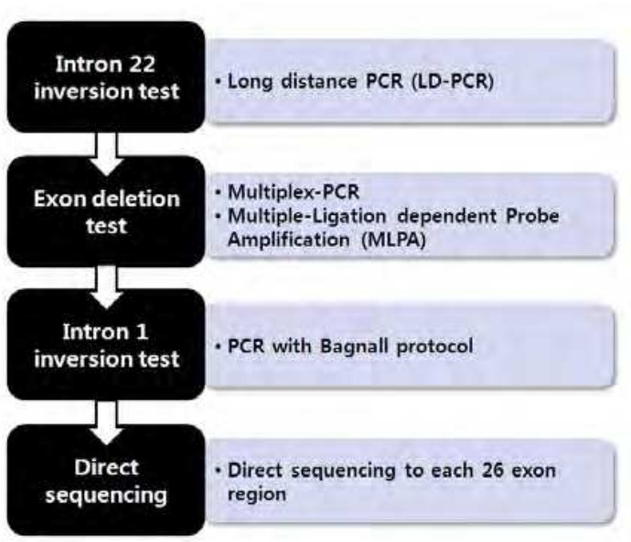

Fig. 1. A proposed strategy for profiling of *F8* mutation.

2.1 Identification of inversions in intron 22 or intron 1

The most common defect in *F8* is intron 22 inversion, which occurs via homologous recombination between *int22h-1* (intragenic) with *int22h-2* or *int22h-3* (extragenic) (Liu et al.,

1998). Figure 2 is a schematic presentation of intron 22 inversion of *F8*. The incidence of intron 22 inversion is approximately 40~50% in severe HA patients, and without a significant ethnic difference (Bowen, 2002). Intron 22 inversion is also a high risk factor for inhibitor formation, thus, it has drawn special attention as a hotspot of *F8* mutation (Oldenburg et al., 2000; Oldenburg et al., 2002). In a previous report, HA patients with intron 22 inversion exhibited an inhibitor prevalence of >22% (Boekhorst et al., 2008). For this reason, tests for intron 22 inversion have been the primary step of *F8* mutation profiling.

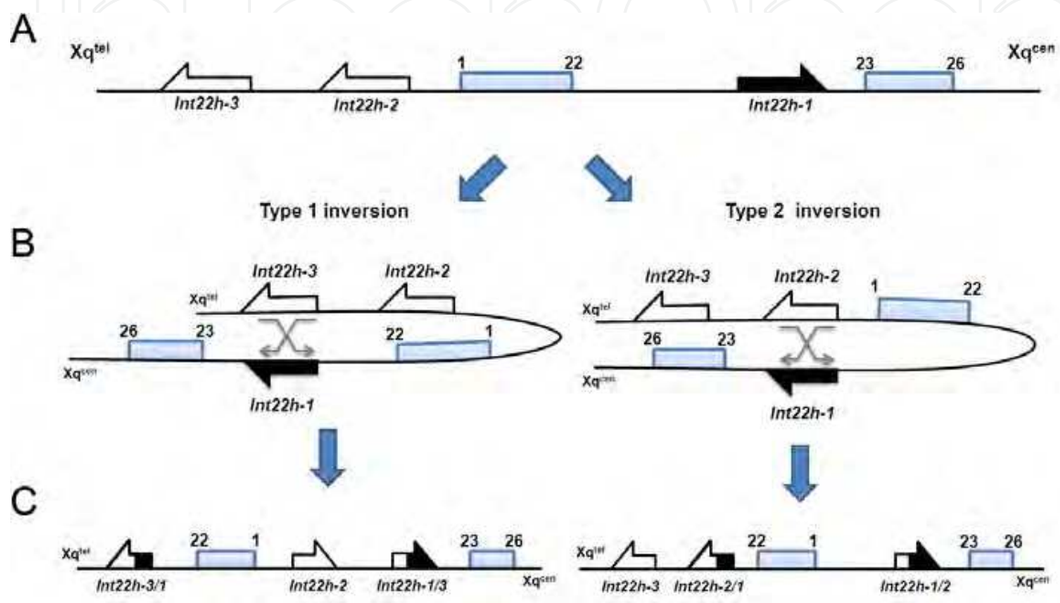

Fig. 2. Schematic presentation of the intron 22 inversion of the *F8*. (A) The normal structure of the *F8* gene. Gray boxes represent exon region and upper number is exon number. White arrow represent intron 22 homologous region (*int22h-2*; proximal and *int22h-3*; distal region) and black arrow indicates *int22h-1* (intragenic). (B) Homologous recombination process occurs between *int22h-1* and *int22h-2* (type 2 inversion) or *int22h-3* (type 1 inversion). (C) The inversions induce disruption of *F8* gene. Exons 1 to 22 are displaced towards the telomere and are oriented in a direction opposite to their normal orientation. Xq^{tel}: X- chromosome q arm telomere, Xq^{cen}: X-chromosome q arm centromere.

Recently, the long-distance PCR (LD-PCR) method was developed for more effective investigation of intron 22 inversion (Liu et al., 1998; Polakova et al., 2003). LD-PCR is conducted with primers P, Q, A, and B in accordance with the methods of Liu *et al* (1998). Primers are designed so that primers P and Q bind to *int22h-1*, whereas primers A and B bind to *int22h-2* and *int22h-3* (Figures 3A and 3B). Figure 3C illustrates an LD-PCR result identifying a Korean HA patient with intron 22 inversion. Lanes 1, 4, and 7 indicate the product of the A+B primer pair (10 kb), which was amplified in both the inversion positive and negative patients. However, there was a difference between the B+P primer pair product in the intron 22 inversion and the wild type; an 11 kb product was generated only in the inversion patient (lanes 5 and 8) but not in the wild type (lane 2). Additionally, the result of the product from P+Q showed that a 12 kb band was generated only in the wild type (lane 3) but not in the inversion patient (lanes 6 and 9). These results demonstrate that the LD-PCR is an effective method for the identification of intron 22 inversion HA patients.

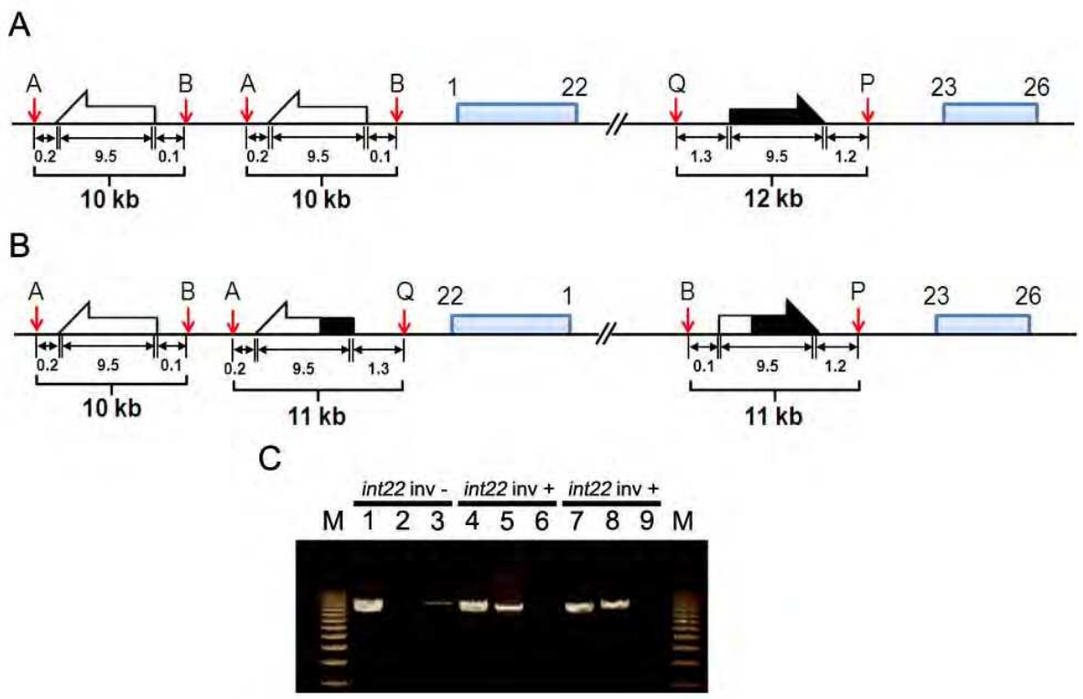

Fig. 3. Primer design for LD-PCR and result of intron 22 inversion test . (A) The normal formation of *F8* gene and intron 22 homologous region and (B) intron 22 inversion-occured *F8* gene. Red arrows represent binding sites for the primers A, B, P and Q. Primers A and B hybridize to forward and rear region of *int22h-2* and *int22h-3*. Combination of primers P and Q hybridize to forward and rear region of *int22h-1*. In the inversion-negative case, LD-PCR with the primers A+B will make a 10 kb PCR product and primers P+Q make a 12 kb one. However, primer B+P will not make any PCR product. While inversion-positive patient will produce a 11 kb band with the primer B+P mixture. (C) Intron 22 inversion test by LD-PCR to one intron 22-negative and two intron 22-positive patients. Lanes 1, 4 and 7 show the results of the primer A+B (product size is a 10 kb) which is amplified in both the inversion and non-inversion cases. Lanes 2, 5 and 8 show the product of the primer B+P mixture for the detection of inversion (11 kb). Lanes 3, 6 and 9 indicate the product of the P+Q primer mixture. M indicates size marker.

Keeney et al (2005) recently reported that multiplex-PCR is available for carrier detection. The multiplex-PCR reaction for the detection of intron 22 inversion is conducted with primers A+B+P+Q combined in 1 tube. If a sister is a HA carrier with intron 22 inversion, 3 bands (10 kb, 11 kb, and 12 kb) will be produced. However, if a sister does not have an intron 22 inversion mutation, the products will be 2 (10 kb and 12 kb) rather than 3 bands.

Similar to intron 22 inversion, intron 1 inversion also occurs via homologous recombination between *int1h-1* (intragenic) and *int1h-2* (extragenic) in the *F8* promoter region (Bagnall et al., 2002). Figure 4 represents a schematic of homologous recombination in *int1h-1* and *int1h-2*. In the figure, homologous recombination will result in an intron 1 breaking inversion and induces a severe mutation. Although several studies have investigated the prevalence of intron 1 inversion, its prevalence remains controversial (1~5% in HA) (Schroder, J. et al.,

2006). The importance of intron 1 inversion is also related to inhibitor formation (Fidanci et al., 2008; Repesse et al., 2007).

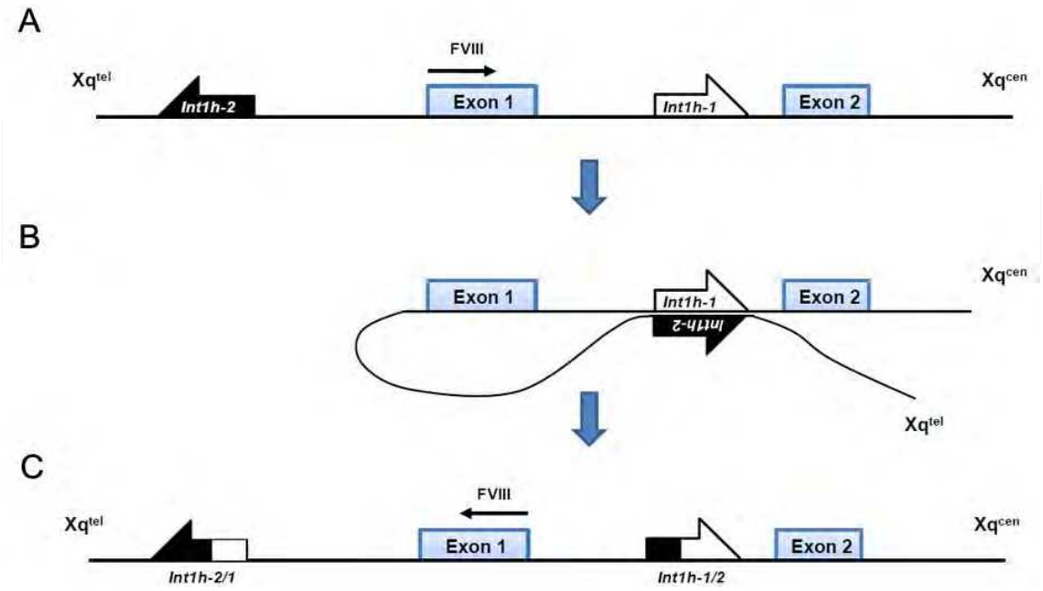

Fig. 4. Scehmatic veiw of intron 1 inversion process. (A) Homologous region of intron 1 is located in the intragenic region (white arrow, *int1h-1*) and extragenic region (black arrow, *int1h-2*). (B) Homologous recombination occurs between *int1h-1* and *int1h-2*. (C) The result of intron 1 inversion will not synthesize an appropriated FVIII protein because the direction of expression is changed. $Xq^{tel:}$ X- chromosome q arm telomere, Xq^{cen}: X-chromosome q arm centromere.

To profile *F8* mutations, we investigated intron 22 inversion and exon deletion (to be discussed later) and then the patients without intron 22 inversion and exon deletion were tested for intron 1 inversion. The amplification products of *int1h-1* and *int1h-2* are analyzed with the method described by Bagnall et al (2002). For detection of intron 1 inversion, primers 9F, 9cF, 2F, and 2R were prepared according to the guidelines established by Bagnall et al (Figure 5). The mixed primers 9cR+9F+2F and 2F+2R+9F were used for the amplification of *int1h-1* and *int1h-2*, respectively. The product of primers 9cR+9F+2F (*int22h-1*) was expected to be a 2.0 kb band, whereas the primers 2F+2R+9F (*int22h-2*) were expected to generate a 1.2 kb product from the wild-type sample (Figure 5A). As shown in Figure 5C, 1.4 kb and 1.8 kb amplicons were produced by the 2F+9F+9cR primers and 2F+2R+9F primers (lanes 1 and 2), respectively, in the case of intron 1 inversion. However, the wild type (inversion test negative) produced 2.0 kb and 1.2 kb PCR products (Figure 5C, lane 3 and lane 4).

2.2 Identification of exon deletion by multiplex-PCR method

Although direct sequencing is a useful method for detection of sequence variation, it has been reported that the method is unable to detect certain gross exon deletions (El-Maarri et al., 2005). For this reason, investigation for gross exon deletion is needed for *F8* mutation profiling before sequence analysis can be carried out. In a previous study, we reported identifying a HA patient with gross exon deletion by applying multiplex-PCR. We designed 35 primers to

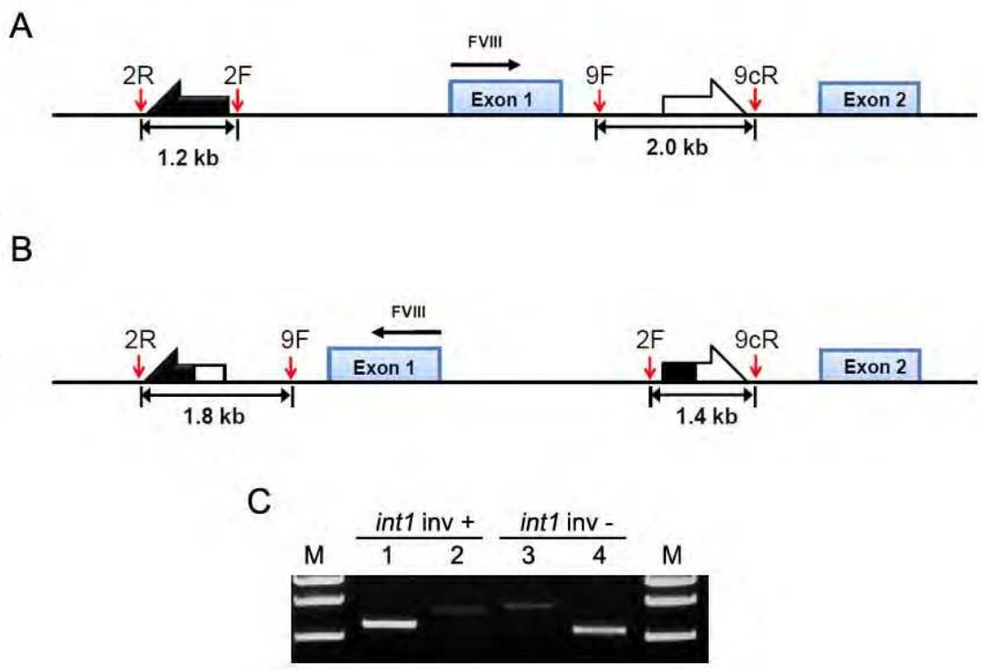

Fig. 5. Primer design and intron 1 inversion test. Linearised diagram of normal (A) and intron 1 inversion (B) of *F8* gene. Red arrows indicate binding sites for each primer. (C) The result of intron 1 inversion test. Primer 2F+9F+9cR combination (lane 1, 3) and primer 2F+2R+9F combination (lane 2, 4) were used for the amplifications of *int1h-1* and *int1h-2*, respectively. (C) Lane 1 and 2 illustrate the product of the intron 1 inversion-positive patient, whereas lane 3 and 4 illustrate intron 1 inversion- negative patients. M: 1 kb size marker.

detect the 26 exons of *F8* (Hwang et al., 2009). In contrast to the routinely used singleplex PCR, which requires 35 PCR reactions per patient to detect exon deletion, only 8 PCR reactions were necessary when multiplex-PCR was used (Figure 6). These results demonstrate that multiplex-PCR is simple and useful for many PCR product analyses in 1-tube reactions. As exon deletion tends to be associated with severe phenotypes, a detection method with simple and accurate application is very important. This method is easily applied to PCR machines and requires no special equipment such as a capillary sequencer for multiplex ligation-dependent probe amplification (MLPA) (Lannoy et al., 2009). Although the MLPA method is powerful and has its advantages, such as being free from primer dimerization and false priming, multiplex-PCR is still a useful method for the detection of exon deletion in local laboratories or in developing countries. Thus, multiplex-PCR analysis can be used as the secondary test prior to direct sequencing. We found that the incidence of gross exon deletion in the Korean HA was 2.6% (Hwang et al 2009).

2.3 Direct sequencing analysis

Finally, direct sequencing can be applied to patients who do not have the mutations mentioned above. In many reports, there is no hotspot for the distribution of sequence variations in *F8* (Bogdanova et al., 2005; Tuddenham et al., 1994). Therefore, all 26 exons, including splicing sites and some portions of the intron region, should be covered by

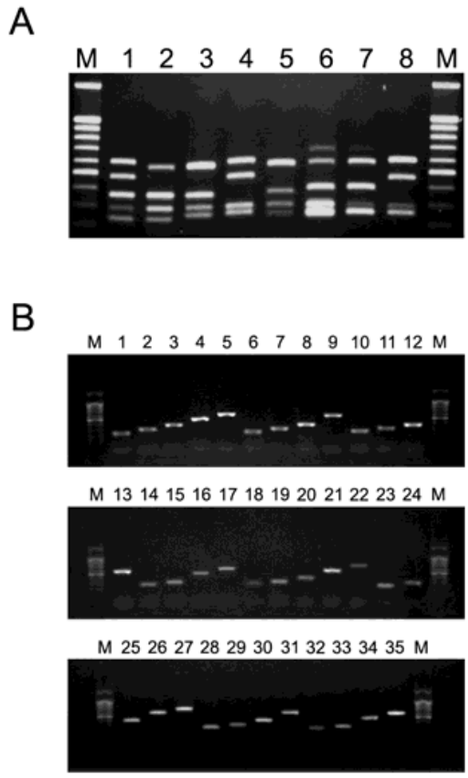

Fig. 6. Detection of gross exon deletion by multiplex-PCR. (A) Multiplex-PCR were performed with 8 primer sets. (B) Singleplex-PCR was performed with 35 primers. The numbers on each lane indicates the primer set (1~8) and single primer (lane 1~35). M: 100 bp size marker.

sequencing analysis. One of the more useful primer sequences is the set developed by David et al (David et al., 1994). These primers contain approximately 20 nucleotides of intronic sequences flanking each exon. The mRNA sequence of *F8* was used for the detection of mutations at splicing sites because certain splicing site mutations are not detected when genomic DNA material is used (Chao et al., 2003; El-Maarri et al., 2005). Conformational sensitive gel electrophoresis (CSGE) or denaturing gradient gel electrophoresis (DGGE) is applied for the detection of mutations with single or larger base mismatches (Korkko et al., 1998). The assay is based on the assumption that a buffer containing mild denaturing solvents can resolve the conformational changes produced by single-base matches in double-strand DNA, which result in an increase of the differential migration in electrophoresis (Korkko et al., 1998). However, these methods are very sensitive to experimental conditions; thus, optimization of conditions is a difficult and time-consuming process. As the cost of sequencing analysis is decreasing by the day, we applied sequencing analysis to each PCR product with reference to the *F8* sequence (NM_000132.3) and without mutation screening by CSGE or DGGE. The results of sequencing were analyzed with diverse programs such as DNASTAR, CLC workbench, and ClusteralW. We identified various sequence variations from Korean HA patients who did not have the mutations mentioned above. These mutations included 8 novel types that were not listed in the HAMSTeRS database (Hwang et al., 2009)

3. Profiling of the *F9* mutation

The identification of disease-causing mutations in the *F9* gene is also critical for diagnosis, genotype-phenotype correlations including inhibitor risk, genetic counseling, and prenatal diagnosis of HB. (Mahajan et al., 2007; Tagariello et al., 2007). More than 1,000 mutations have been reported in the literature, and the distribution of mutation types in HB is somewhat different from those in HA (HGMD Professional 2010.4, release date 18 December 2010, URL: http://www.hgmd.org/). A locus-specific mutation database also exists for HB (The Hemophilia B Mutation Database – version 13, last update in 2004, URL: http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html). Point mutations account for the majority of mutations (~90%) and large exon deletion mutations account for ~6%. Complex rearrangement mutations without exonal dosage changes (copy-neutral) have rarely been reported; large inversion mutations such as intron 22 inversion in HA have not been reported in HB. Missense/nonsense mutations account for ~70% of point mutations, followed by small insertion/deletion mutations (~17%). In addition, it is notable that whole gene deletions account for approximately half of the large exon deletion mutations in *F9*. Based on the line of evidence collected from the literature and mutation database, the following is a proposed procedure for profiling *F9* mutations (Figure 7).

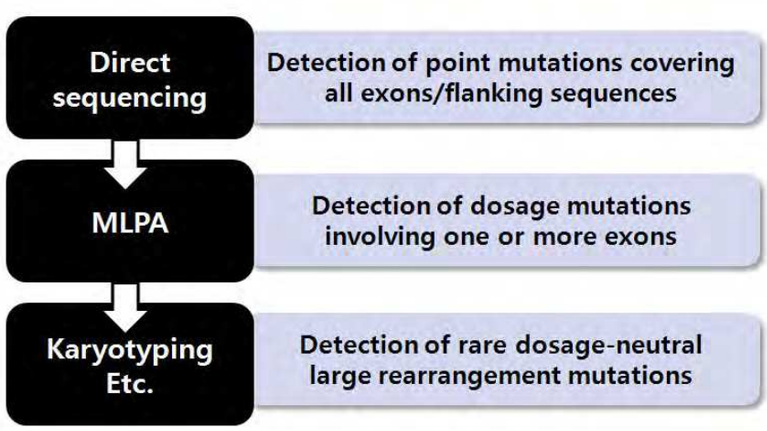

Fig. 7. A proposed strategy of *F9* mutation profiling.

3.1 Identification of *F9* point mutations by direct sequencing analysis

As point mutations account for ~90% of cases, direct sequencing can be the first-line diagnostic modality for molecular diagnosis in HB. As in HA, the mutations are scattered throughout the gene, thus, sequencing analyses need to cover the coding sequences and flanking intronic sequences of all 8 exons (Kwon et al., 2008). The strategy for direct sequencing analysis is largely similar to that for HA, but is simpler and less costly because the *F9* gene is smaller and is encoded by a smaller number of exons. In addition, as in HA, mutation scanning by CSGE can also be applied for direct sequencing analyses, but the detection sensitivity of CSGE needs to be validated in each laboratory prior to clinical implementation (Santacroce et al., 2008). Large deletion mutations, which can be detected by MLPA analyses, should be suspected when 1 or more reactions to amplify a genomic segment fail. Below is an example of a sequencing result with a missense mutation in a Korean HB (Kwon et al., 2008).

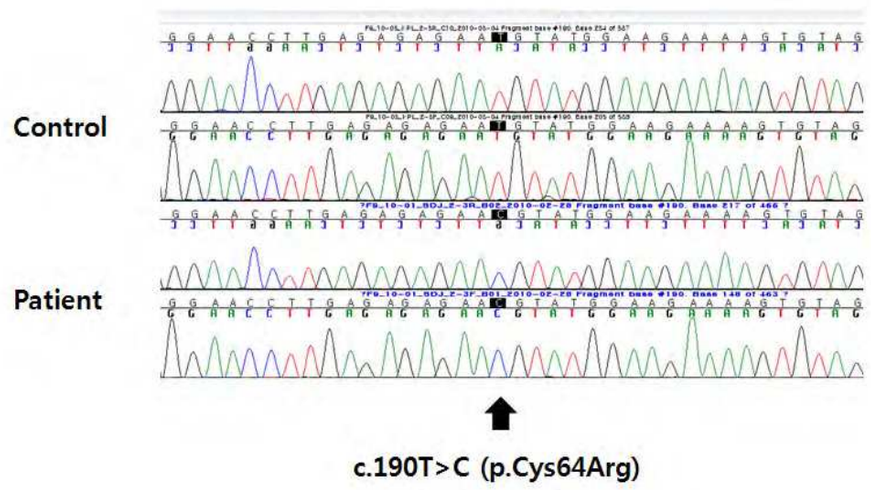

Fig. 8. A point mutation (missense mutation) leading to the substituion of the 64th amino acid residue cysteine to arginine detected by direct sequencing analyses in a Korean male patient with HB

3.2 Identification of large exon deletion mutations by multiplex ligation-dependent probe amplification

The possibility of large exon deletion mutations should be considered (second-line molecular genetic test in HB) when no point mutations are identified through direct sequencing analyses or when PCR experiments fail on 1 or more exons. As in HA, the MLPA technique is a robust molecular test to detect mutations of large exon deletion affecting 1 or more exons in *F9* (Kwon et al., 2008). The principle and method of interpretation of MLPA results are similar to that for *F8*. The detection of this type of mutation is particularly important since it implicates a high risk of inhibitor development (Giannelli et al., 1983; Oldenburg et al., 2004). The real-time quantitative PCR technique can also be used to detect large exon deletion mutations in *F9* (Vencesla et al., 2007). However, recent studies have pointed out the advantages of MLPA over real-time PCR (Casana et al., 2009). Figure 9 is an example of a result of a multiplex ligation-dependent probe amplification experiment with whole gene deletion in a Korean male HB.

3.3 Identification of large rearrangement mutations without large exon deletion changes

The need to search for copy-neutral large rearrangement mutations arises when no point mutations or large exon deletion mutations are detected on direct sequencing followed by MLPA analyses. In particular, a balanced chromosomal rearrangement involving the *F9* gene on the Xq27.1 band disrupts the normal transcription and translation of the molecule, leading to HB. Karyotype analyses using peripheral blood lymphocytes can detect rearrangements such as t(X;1)(q27.1;q22 or q23) and t(X;15)(q27.1;p11.2) (Ghosh et al., 2009; Schroder, W. et al., 1998). In particular, these rearrangements can be the genetic backgrounds of female HB with or without family history. X chromosome analyses are needed in such cases to confirm skewed inactivation of the non-rearranged copy of the X chromosome.

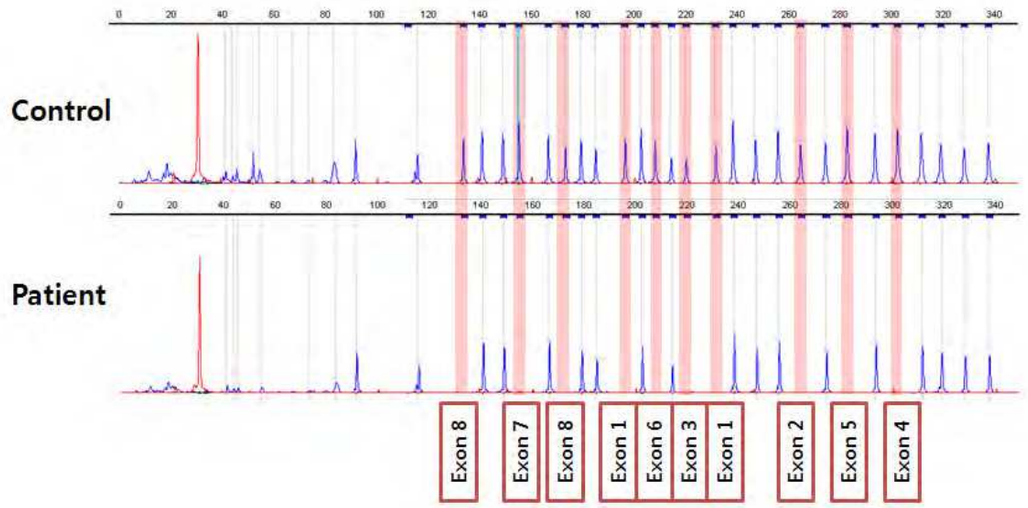

Fig. 9. The chromatographic results of the multiplex ligation-dependent probe amplificaiton experiment showing the whole *F9* gene deletion in a male patient with haemophilia B

4. New approach of the mutation profiling

Technologies for more efficient detection of mutations such as microarrays and next generation sequencing (NGS) have been developed. Although mutation testing with microarrays has received attention, it faces limitations in identifying various mutations (Berber et al., 2006; Chan et al., 2005). In addition, microarray-identified mutations require validation to eliminate false positive or false negative results (Johnson et al., 2010). On that point, NGS is a prospective approach in *F8* mutation studies (Lindblom & Robinson, 2011). NGS is an alternative sequencing strategy that redefines "high-throughput sequencing". These technologies outperform the older Sanger-based sequencing by throughput capacity and reduce the cost of sequencing. However, NGS still faces some problems in application to *F8* or *F9* sequencing for mutation identification. The cost of NGS equipment is more expensive than that of other capillary sequencing machines. As NGS sifts through a large amount of data, a bioinformatics expert is needed to analyze the high-throughput sequencing data. Recently, NGS companies have begun launching mini-scale (personal sequencing system) equipment.

Typical examples of mini-scale NGS machines are the GS junior system from Roche, which is based on 454 sequencing, the MiSeq from Illumina, and the Ion torrent from Life Technology. These equipments can amplify 10–100 M genes with proven technology (Glenn, 2011). Moreover, they can be applied variously to amplicon sequencing assays, small genome sequencing, exome sequencing, and genome-wide association study (GWAS) targeted regions (Grossmann et al., 2011). They also require neither bulky equipments for analysis nor lengthy time to produce a large amount of results. These advantages of mini-scale sequencing are considered useful for the identification of *F8* or *F9* sequence variants. Established capillary electrophoresis requires at least 40 reactions to analyze the 26 exons in the *F8* gene from 1 person. It would take approximately 3,840 sequencing reactions to survey 96 patients for the *F8* mutation (Grossmann et al., 2011). This uses a lot of money and

is labor intensive. However, the MiSeq system and TruSeq® amplicon sequencing method requires just 1 sequencing reaction to carry out the task and a week to analyze *F8* sequence variations. This prospective tool could be widely used in hemophilia diagnosis.

5. Concluding comments

Mutations in *F8* result in truncated FVIII proteins, which can affect their interaction with other proteins in the coagulation cascade. Some mutations affect the recognition region of molecular chaperone proteins in the Golgi apparatus or endoplasmic reticulum during post-translational modification of FVIII (Dorner et al., 1987; Lenting et al., 1998; Leyte et al., 1991). Another consideration of the *F8* or *F9* mutation is closely related with the development of inhibitory antibodies. For these reasons, effective profiling of mutations in *F8* or *F9* is important for the diagnosis and therapy of hemophilia, as well as prediction of inhibitor development.

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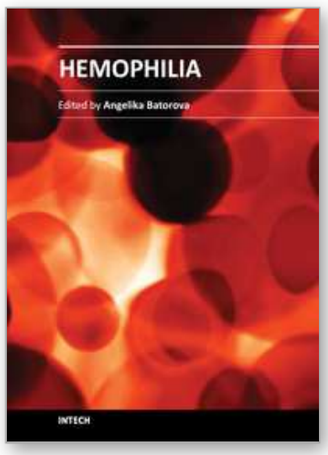

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This book demonstrates the great efforts aimed at further improving the care of the hemophilia, which may bring further improvement in the quality of life of hemophilia persons and their families.

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