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PRAPAPORN THANOOTARAKUL : DETECTION OF MUTATIONS IN THE FACTOR VIII GENE BY mRNA ANALYSIS. THESIS ADVISORS : PATHAI YENCHITSOMANUS, Ph.D., VARAPORN AKKARAPATUMWONG, Ph.D.
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Hemophilia A is an X-linked bleeding disorder caused by deleterious mutations in the coagulation factor VIII gene. The factor VIII gene located at Xq28 and spanned 186 kb, is composed of 26 exons and transcribes mRNA with the length of about 9 kb. The molecular defects of factor VIII gene causing hemophilia A are heterogeneous, giving rise to clinical heterogeneity. The characterization of pathogenic mutations of the factor VIII gene allows the recognition of new mechanisms of functional disturbances of factor VIII. The molecular characterization of mutations in Thai hemophilia A patients in this study was carried out by the methods of reverse transcription-polymerase chain reaction (RT-PCR), single strand conformation polymorphism (SSCP), and direct DNA sequencing. The RT-PCR method for amplification across the inversion break-point region was used to screen the factor VIII gene inversion, which separates exons 1-22 from exons 23-26 and is found to be a common cause of severe hemophilia A. A combination of long RT-PCR of the factor VIII mRNA and PCR of factor VIII genomic DNA were used to isolate the factor VIII sequence for screening small molecular defects. The entire factor VIII cDNA sequences were amplified and fractionated into seven overlapping fragments by nested PCR. The exon 14 was amplified into two overlapping fragments either by using long cDNA or genomic DNA as a template. The fragments were digested with appropriate restriction enzymes to generate the size suitable for SSCP. DNA sequencing was used to identify mutations detected by the SSCP screening. Once the mutations were identified, the allele-specific amplification (ASA) was developed and used for analysis in all available members of the family to confirm the identification and examine the carrier status.

RT-PCR analysis for the gene inversion of 11 samples from severe hemophilia A patients revealed the discontinuity of RNA when a PCR on reverse transcribed RNA was performed across exons 22-23, demonstrating the presence of factor VIII gene inversion. Ten of these patients have been investigated to have gene inversions by Southern-blot hybridization. The RT-PCR method developed is useful for screening factor VIII gene inversion. cDNA analysis of 18 hemophilia A patients revealed the skipping of total 154 bp of exon 15 combined with the absence of the first 47 bp of exon 16 in one patient due to the G to T transversion at the donor splice site of intron 15 and the activation of a cryptic acceptor splice site within exon 16. The SSCP analysis was performed in 8 hemophilia A patients from 6 families. The mobility shifts were observed in the same cDNA fragment in 2 patients of the same family. From DNA sequencing, it was found that there was a nucleotide substitution at codon 233, ACA>ATA, in exon 6 of the factor VIII gene. This mutation resulted in a novel missense mutation (T233I) in the A1 domain. These results suggest that carrier status in members of both families could be detected by the ASA technique.