Heterogeneous lengths of copy number mutations in human coagulopathy revealed by genome-wide high-density SNP array

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ABSTRACT

Background
The recent advent of genome-wide molecular platforms has facilitated our understanding of the human genome and disease, particularly copy number aberrations. We performed genome-wide single nucleotide polymorphism-array in hereditary coagulopathy to delineate the extent of copy number mutations and to assess its diagnostic utility.

Design and Methods
The study subjects were 17 patients with hereditary coagulopathy from copy number mutations in coagulation genes detected by multiple ligation-dependent probe amplification. Eleven had hemophilia (7 hemophilia A and 4 hemophilia B) and 6 had thrombophilia (4 protein S deficiency and 2 antithrombin deficiency). Single nucleotide polymorphism-array experiments were performed using Affymetrix Genome-Wide Human SNP arrays 6.0.

Results
Copy number mutations were identified by single nucleotide polymorphism-array in 9 patients, which ranged in length from 51 Kb to 6,288 Kb harboring 2 to ~160 genes. Single nucleotide polymorphism-array showed a neutral copy number status in 8 patients including 7 with either a single-exon copy number mutation or duplication mutations of *PROS1*.

Conclusions
This study revealed unexpectedly heterogeneous lengths of copy number mutations underlying human coagulopathy. Single nucleotide polymorphism-array had limitations in detecting copy number mutations involving a single exon or those of a gene with homologous sequences such as a pseudogene.

Key words: coagulation, contiguous gene deletion, copy number, hemophilia, mutation, single nucleotide polymorphism-array, thrombophilia.

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Introduction

The application of high-throughput genome-wide analysis of genetic variations has facilitated our understanding of the human genome and the genotype-phenotype correlations.[1-4] The variability of the human genome is largely determined by 2 types of variations: single nucleotide variations and copy number (CN) variations. CN variations represent genomic segments with a size range of ~100 bp to several Mbs demonstrating an altered (or non-neutral) dosage status.[5] They are typically introduced in the genome by recombination-based or replication-based mechanisms. The detection of CN variations is clinically important because they can provide the genetic background of human diseases, including Mendelian disorders, contiguous gene deletion syndromes, and common complex diseases.[6-8] Two representative platforms to detect CN variations are array comparative genomic hybridization (CGH) and single nucleotide polymorphisms (SNP)-array. SNP-array produces genotype data from up to one million SNPs over the genome determined by the hybridization signals. The SNP-array platform has been proven to be a robust genome analysis tool not only to obtain genotype data but also to detect CN variations, uniparental disomy, and consanguinity.[9]

Hereditary coagulation disorders cover a variety of congenital deficiencies of proteins involved in the physiological balance of the coagulation system. Depending on the molecule involved, the clinical manifestation can be either hemophilic (pathological bleeding) or thrombophilic (pathological clotting). Deficiencies of coagulation factor VIII (FVIII, hemophilia A [HA]; MIM 306700) and factor IX (FIX, hemophilia B [HB]; MIM 306900) from mutations in the *F8* and *F9* gene, respectively, are one of the representative human Mendelian disorders, with X-linked recessive inheritance.[10] On the other hand, deficiencies of natural anticoagulants, protein C (PC def, MIM 176860), protein S (PS def, MIM 612336), and antithrombin (AT def, MIM 613118), from mutations in *PROC*, *PROS1*, and *SERPINC1*, cause hereditary thrombophilia. Traditionally, the molecular diagnosis of these hereditary coagulation disorders has focused on the detection of point mutations by sequencing analyses, except for the inv(22) mutation of *F8* in HA. However, the recent introduction of technically improved platforms for targeted CN mutation analysis, such as multiplex ligation-dependent probe amplification (MLPA), have revealed that CN mutations involving one or more exons to the whole gene are more common than has been recognized in hereditary coagulopathy.[11,12] Moreover, it was reported that the deleted region involved unexpectedly large genomic regions outside the coagulation gene of interest.[13]

Given this, we performed high-throughput genome-wide SNP-array experiments in a series of patients with hereditary coagulopathy to delineate the extent of causative CN mutations and to assess its diagnostic utility.

Design and Methods

Study subjects

The study subjects were 17 patients with hereditary coagulopathy with CN mutations in the causative coagulation genes. Patients' characteristics are shown in Table 1. The hemophilia (bleeders) group consisted of 10 patients with hemophilia and one hemophilia carrier (Table 1, H1-H11). Seven patients with HA (H1-H7) had severe FVIII deficiency including 5 who were inhibitor-positive. Patient H7 was a 5-year old girl with severe HA who had been documented to have inherited inv(22) mutation of *F8* from her father with severe HA. Three patients with HB (H8-

Table 1. Seventeen study patients with hereditary coagulation disorders with copy number mutations identified by MLPA analyses.

Patient	Age/sex	Clinical Dx	Coag Dx	Gene	MLPA	Inhibitor
H1	5/M	Bleeder	HA, severe	*F8*	Del, exon 7-26[14]	(+)
H2	2/M	Bleeder	HA, severe	*F8*	Del, exon 1-22	(+)
H3	24/M	Bleeder	HA, severe	*F8*	Dup, exon 24	(−)
H4	26/M	Bleeder	HA, severe	*F8*	Del, exon 16-19	(+)
H5	43/M	Bleeder	HA, severe	*F8*	Del, exon 24	(+)
H6	12/M	Bleeder	HA, severe	*F8*	Del, exon 1	(+)
H7*	5/F	Bleeder	HA, severe	*F8*	Del, WG[15]	(−)
H8	5/M	Bleeder	HB, severe	*F9*	Del, exon 1[16]	(+)
H9	3/M	Bleeder	HB, severe	*F9*	Del, WG	(−)
H10	2/M	Bleeder	HB, severe	*F9*	Del, WG	(−)
H11	60/F	ASx	HB, carrier	*F9*	Del, WG	N/A
T1	39/M	PVT	PS def.	*PROS1*	Dup, exon 5-10[17]	N/A
T2	44/F	DVT & PE	PS def.	*PROS1*	Del, exon 1[18]	N/A
T3	47/M	VTE	PS def.	*PROS1*	Del, exon 2	N/A
T4	25/M	ASx	PS def.	*PROS1*	Dup, exon 5-10	N/A
T5	27/M	DVT & PE	AT def.	*SERPINC1*	Del, exon 1-6[19]	N/A
T6	49/M	DVT & SMVT	AT def.	*SERPINC1*	Del, exon 6[19]	N/A

*MLPA: multiplex ligation-dependent probe amplification; Dx: diagnosis; Coag: coagulation; HA: hemophilia A; HB: hemophilia B; ASx: asymptomatic; Del: deletion; Dup: duplication; WG: whole gene; N/A: not applicable; def: deficiency; PVT: portal vein thrombosis; DVT: deep vein thrombosis; PE: pulmonary embolism; SMVT: superior mesenteric vein thrombosis. *Also had inv(22) along with the whole gene deletion of F8.[15]*

H10) had severe FIX deficiency including one who was inhibitor-positive (H8). Individual H11 was a female obligatory carrier from a family with HB and was tested for prenatal diagnosis. The thrombophilia (clotters) group consisted of 5 patients with venous thromboembolism (T1-T3, T5, and T6) and one asymptomatic individual (T4) who was screened for coagulopathy as a donor for liver transplantation. Four had protein S deficiency based on functional coagulation tests (T1-T4), and 2 had antithrombin deficiency (T5 and T6). The possibility of CN mutations was considered in these 17 subjects based on the observation of negative point mutations in the coding exons and flanking sequences of the target coagulation genes on direct sequencing analyses. Particularly in male patients with hemophilia, PCR failure of one or more exons of *F8* and *F9* genes on the X chromosome strongly indicated exon deletion mutations and prompted us to proceed with MLPA experiments to detect CN mutations.

MLPA analyses

Written informed consent was obtained from all patients or guardians for molecular genetic diagnosis under the approval of the Institutional Review Board. Genomic DNA was extracted from peripheral blood leukocytes using the Wizard Genomic DNA Purification Kit following the manufacturer's instructions (Promega, Madison, WI, U.S.A.). MLPA experiments were performed using commercial kits SALSA MLPA P178 F8, P207 F9, P265 PROS1, and P227 SERPINC1 (MRC-Holland, Amsterdam, The Netherlands) as described previously.[16] The results of MLPA analyses in 7 of the study patients had been reported previously (Table 1).

SNP-array analyses

For the SNP-array experiment, DNA concentration and purity were determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). The DNA samples were genotyped by using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, each of 250 ng of DNA samples was digested with *Nsp* I and *Sty* I enzymes, followed by adaptor-ligation and amplification. The amplified DNA was then fragmented, labeled, and hybridized to the array. The arrays were then washed, scanned, and the image data were analyzed and compared with the data from control DNA samples using the Hidden Markov Model on the Genotyping Console 3.0.2 (Affymetrix). The UCSC Genome Browser Build hg18/NCBI Map Viewer Build 36.3 was used to obtain information on the genomic segments involved in the CN mutations detected and the corresponding list of genes.

Results

CN mutations in hemophilia genes

Eleven patients/carrier with hemophilia had CN mutations involving a single exon to the whole gene identified by MLPA analyses (Table 1, Patients H1-H11). CN mutations of *F8* in 7 patients with HA showed a heterogeneous range of segments involved, with only one patient (H7) with a whole gene deletion (14%; 1 of 7). On the other hand, CN mutations of *F9* in 4 patients/carrier of HB were mostly whole gene deletion (75%; 3 of 4). Both in HA and HB, CN mutations were mostly deletions (deletion in 10 *vs.* duplication in one). SNP-array experiments could successfully delineate the segments of CN mutations in 7 cases out of 10 with successful results (70%). The SNP-array data were not successfully obtained in H4, possibly from the poor quality of specimen. The length of CN mutations in hemophilia revealed by SNP-array ranged from ~51 Kb (H6) to ~6,288 Kb (H7) (Table 2 and Figure 1). In all cases, the CN mutation involved other neighboring genes, from a single gene (*FUNDC2* in H6) to ~161 genes (H7), compatible with contiguous gene deletion (Table 2, Figure 1, and *Online Supplementary Figure S1*, A-E). The SNP-array experiment showed a neutral CN status in 3 patients with hemophilia: Patient H3 with duplication of exon 24 of *F8*, Patient H5 with deletion of exon 24 of *F8*, and Patient H8 with deletion of exon 1 of *F9* (*Online Supplementary Figure S2*, A-C).

CN mutations in thrombophilia genes

Four patients with PS deficiency were shown to have CN mutations of *PROS1* by MLPA, 2 with duplication of exons 5-10 (Table 1, T1 and T4) and one each with deletion of exon 1 (T2) and exon 2 (T3). Two patients with AT deficiency had deletion of exon 1-6 (T5) and deletion of exon 6 (T6), respectively. SNP-array experiments could successfully delineate the segments of CN mutations in 2 cases out of 6 (33%) both of which involved other neighboring genes (Table 2). In Patient T2 with deletion of exon 1 of *PROS1* on MLPA, SNP-array showed a deletion segment of ~67 Kb including the adjacent *ARL13B* gene

Table 2. Copy number mutations identified by SNP-array in 9 patients with hereditary coagulopathy.

Patient	MLPA	Loss/ gain	Chromosome band	Size (Kb)	Start marker	Start position	End marker	End position	N. of markers	N. of genes*
H1	*F8*_E7-26_del[21]	Loss	q28	~157	CN_921539	153703648	CN_923648	153860284	68	5 (3**)
H2	*F8*_E1-22_del	Loss	q28	~283	CN_923610	153755068	CN_923710	154038338	118	7 (6**)
H6	*F8*_E1_del	Loss	q28	~51	CN_923660	153891415	CN_404047	153942523	15	2 (2**)
H7***	*F8*_WG_del	Loss	q28	~6,288	CN_927985	148598983	SNP_A-8528843	154887040	3,307	161 (126**)
H9	*F9*_WG_del	Loss	q27.1	~797	CN_932113	137863600	CN_940934	138660733	581	13 (7**)
H10	*F9*_WG_del	Loss	q26.3-q27.3	~5,739	CN_932026	137633209	CN_930036	143372580	3,706	42 (28**)
H11	*F9*_WG_del	Loss	q26.3-q27.2	~2,822	CN_932058	137702377	CN_912446	140524120	1,686	27 (16**)
T2	*PROS1*_E1_del[20]	Loss	3q11.2	~67	CN_1029570	95160010	CN_1031689	95226611	26	4 (3**)
T5	*SERPINC1*_E1-6_del[22]	Loss	1q25.1	~66	SNP_A-2092517	172143328	CN_009040	172209802	42	2 (2**)

*SNP: single nucleotide polymorphism; Coag: coagulation; MLPA: multiplex ligation-dependent probe amplification; del: deletion; dup: duplication; WG: whole gene. *See Online Supplementary Table 1 for the list of genes. **Only including unique functional genes approved by the HUGO Gene Nomenclature Committee (HGNC). ***Also had inv(22) along with the whole gene deletion of F8.[15]*

(*Online Supplementary Figure S1F*). In Patient T5 with deletion of exons 1 through 6 of *SERPINC1* on MLPA, SNP-array showed a deletion segment of ~66 Kb including the adjacent *RC3H1* gene (*Online Supplementary Figure S1G*). SNP-array showed a neutral CN status in 2 patents with duplication of exons 5-10 of *PROS1* (T1 and T4), Patient T3 with exon 2 deletion of *PROS1*, and Patient T6 with deletion of exon 6 of SERPINC1 (*Online Supplementary Figure S2 D-G*).

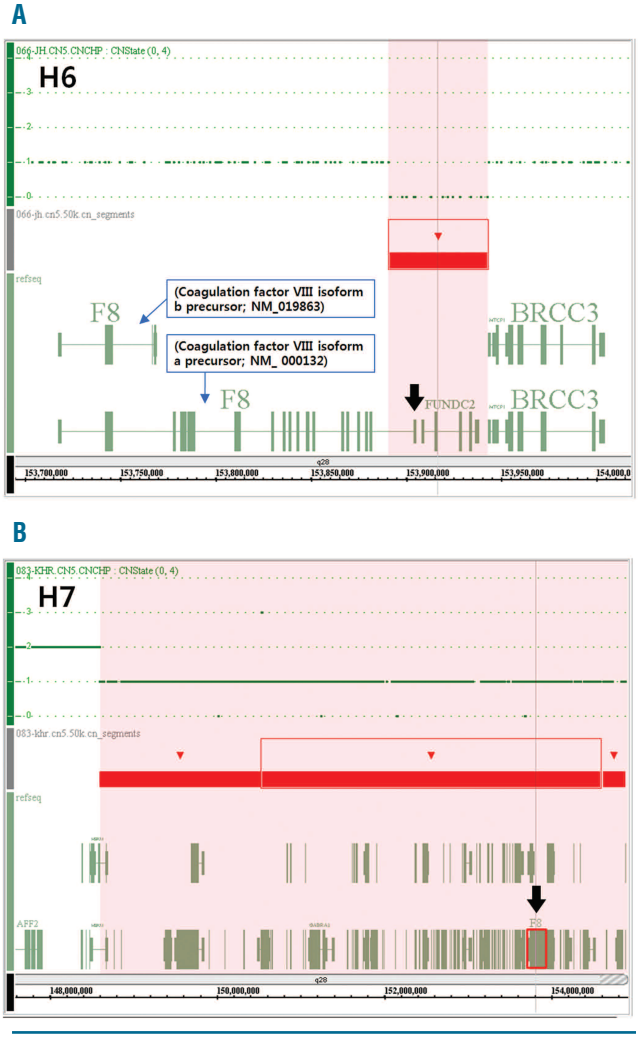

Figure 1. (A) The screenshots of the Genotyping Console representing the SNP-array results that showed copy number (CN) mutations causing hereditary coagulation disorders. The customized screen consists of 4 parts: from top to bottom, 1) the CN status from target loci (0 to 4; green dots or bars), 2) the genomic segment with CN aberrations detected (thick red bars; arrowheads indicate CN loss), 3) refseq (reference sequence) genes in the corresponding genome), and 4) the idiogram and location of the region. CN of 1 and 2 represent the neutral status for X chromosome in male and female individuals, respectively. Also note that transcript variants are shown in the refseq panel (for example, NM_000132 and NM_019863 for F8 as indicated by blue boxes). (A) Patient H6 with severe hemophilia A with ~51 Kb deletion (CN 0; the genomic segment shaded in light red) involving the exon 1 of *F8* (thick black arrow). Note that the adjacent *FUNDC2* gene is also included in the deletion segment. (B) Female Patient H7 with severe hemophilia A showing ~6,299 Kb deletion (CN 1; the genomic segment shaded in light red) involving the whole gene of *F8* (small red box in the bottom indicated by a thick black arrow) and 161 neighboring genes. She also had inv(22) mutation of paternal origin.

Discussion

In this study, SNP-array experiments demonstrated unexpectedly heterogeneous lengths of segments of CN mutations underlying hereditary coagulopathies of Mendelian inheritance, ranging from 51 Kb to 6.3 Mb. Notably, in all 9 patients with CN mutations detected by SNP-array, the segment of CN mutation involved other neighboring genes, from 2 to 161 genes (contiguous gene deletion) (Table 2 and *Online Supplementary Table S1*). In the hemophilia group (Table 1, Patients H1-H11), whole gene deletion was more frequent in HB (H8-H11, 75%) than in HA (H1-H7, 14%). In comparison with the hemophilia group, the deletion segments were relatively short in the thrombophilia group; half the patients had a single exon aberration and only 2 patients had CN mutations involving one or more neighboring gene (Table 2, T2 and T5). Particularly in thrombophilia, recent studies have shown that large CN mutations involving one or more exons are particularly relevant to PS deficiency.[12] In line with this observation, PS deficiency from CN mutations of *PROS1* accounted for the majority of our series of thrombophilia cases (67%), and AT deficiency from *SERPINC1* mutations accounted for the rest (33%).

As for the genotype-phenotype correlations, we correlated the severity of disease and inhibitor development in hemophilia cases with the genetic defect identified. HA and HB are categorized as severe, moderately severe, and mild, based on the residual factor activity. All patients with HA/HB in our series had the severe phenotype (residual factor activity <1%), which is in line with the previously acknowledged genotype-phenotype correlation that large gene rearrangement mutations are associated with severe disease. Approximately 20-30% of patients with severe HA and 3% of HB develop inhibitors to defective coagulation factor, which is the most serious and challenging complication.[20,21] Large, multi-domain CN mutations are known to be the single most significant risk factor to inhibitor development with a frequency of up to 80% in HA.[22] Indeed, the frequency of inhibitor in our series of large CN mutation was 71% in HA (5 of 7) and was 33% in HB (1 of 3) (Table 1). Two patients with HA without inhibitor were a male patient with a single exon duplication (H3) and a female patient with HA (H7). The patient with HB who had high-titer inhibitor (H8) was previously reported to be the only patient with inhibitor among 33 HB patients.[16] Two patients with HB who were negative for inhibitor were a 2- and 3-year old boy, respectively, and thus careful management is mandatory in these young, high-risk patients.

Regarding the phenotypic contribution of the CN aberration in neighboring genes, no patient among the 9 had definite phenotypic abnormalities other than coagulation defect (Table 2). The nullisomic deletion of neighboring genes in hereditary coagulopathy was first reported in 2 unrelated patients with HB back in 1988.[13] These patients were inhibitor-positive and had a large deletion encompassing *F9* and *MCF2* genes on the X chromosome. Of note, they had no clinical condition attributable to the loss of *MCF2*. In all 3 cases of HB with CN mutations by SNP-array, the segments included *MCF2* (*Online Supplementary Table S1*). Patient H7, a female patient with HA, was compound heterozygous for inv(22) and the large CN mutation and demonstrated completely skewed X chromosome inactivation status.[15] The CN mutation involved a

genomic segment longer than 6 Mb harboring 161 genes (Figure 1B). Cytogenetic analysis was not performed in the patient. A CN aberration involving a genomic segment with a size 5-10 Mb can be detected on cytogenetic analyses. However, the CN mutation in Patient H7 would not be readily visible because it is on a light band of the tip of X chromosome (Xq28) on conventional G-bands. Family study revealed that the X chromosome with the large CN mutation was inherited from her carrier mother, but there was no history of HA on the maternal side. We could not determine whether the large deletion was a *de novo* occurrence in her mother or not, since no further family study was performed on the maternal side. We speculated that a male individual with the large contiguous gene deletion might have other clinical manifestations in addition to HA. In addition, a meticulous and targeted phenotypic assessment could reveal relevant clinical and/or laboratory findings in at least some of our series of patients with hereditary coagulopathy with contiguous gene deletion.

In 7 patients out of 16 (44%; not including Patient H4 with unsuccessful SNP-array results), SNP-array showed neutral CN status in the region of interest *(Online Supplementary Figure S2*). The CN mutations in these patients were either deletion/duplication of a single exon (Table 1, H3, H5, H8, T3, and T6) or duplication mutation of *PROS1* (T1 and T4).[17] A total of 7 patients in our series (41%) had a CN mutation involving a single exon of the coagulation gene, exon 1 accounting for 43% (3 of 7). The segment of CN mutations in 2 of these 3 with exon 1 aberration involved neighboring genes. The failure to detect CN mutation in small segments involving a single exon, particularly when it was not exon 1, could be due to the limited coverage of SNP markers in the region of interest. The single exon involved in CN mutation was not exon 1 in all patients with neutral CN status by SNP-array, except in Patient H8. On the other hand, the duplication mutation of exons 5-10 of *PROS1* in Patients T1 and T4 were not detected by SNP-array. The SNP-array used in this study has 5-9 SNP markers in this region, which clearly revealed neutral CN status (*Online Supplementary Figure S2D and F*). The duplication mutation was previously proven by sequencing analysis.[17] In these 2 cases with PS deficiency, the presence of the pseudogene *PROSP*, with a high sequence homology with *PROS1* (exons 5-10 of *PROS1* correspond to exons 1-6 of *PROSP*) could be a possible mechanism underlying the discrepancy between MLPA and SNP-array results.

As demonstrated in the results of this study, MLPA analysis can be the first-line test to detect CN mutation in single-gene disorders, since every single exon is specifically targeted in a gene of interest. However, SNP-array can reveal the extent of CN mutation outside the gene, and can also narrow down the rearrangement breakpoints. In addition, genomic information other than local CN status can be obtained and used for other purposes; for example, association studies for disease susceptibility. Lastly, the cost of SNP-array has been rapidly decreasing recently, which must also be taken into account when considering its diagnostic utility. The major disadvantage of SNP-array is the limited coverage (sensitivity) and specificity. A particular gene of interest might not be densely covered enough to detect or precisely identify the CN mutation in the gene (as was the case in Patients H3, H5, H8, T3, and T6). In addition, results cannot be reliably obtained in targets with high homologous sequences such as pseudogenes (as in Patients T1 and T4). To overcome these limitations, more recently targeted SNP-arrays and exon-focused or custom array-CGH have been developed.

In conclusion, this study is the first investigation on the large CN mutations underlying hereditary coagulation disorders in human using high-resolution SNP-array analyses. SNP-array revealed deletion or duplication mutations involving a single exon of coagulation genes to contiguous gene deletion. We believe the data from genome-wide SNP-array in hereditary coagulation disorders would extend our knowledge on genotype-phenotype correlations and susceptibility of large CN mutations by fine delineation of the boundaries of rearrangements.

Authorship and Disclosures

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