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Germline Copy Number Variations in *BRCA1*-Associated Ovarian Cancer Patients

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We investigated characteristics of germline copy number variations (CNV) in *BRCA1*-associated ovarian cancer patients by comparing them to CNVs present in sporadic ovarian cancer patients. Germline CNVs in 51 *BRCA1*-associated, 33 sporadic ovarian cancer patients, and 47 healthy women were analyzed by both signal intensity and genotyping data using the Affymetrix Genome-Wide Human SNP Array 6.0. The total number of CNVs per genome was greater in the sporadic group (median 26, range 12–34) than in the *BRCA1* group (median 21, range 11–35; *post hoc* $P < 0.05$) or normal group (median 20, range 7–32; *post hoc* $P < 0.05$). While the number of amplifications per genome was higher in the sporadic group (median 13, range 7–26) than in the *BRCA1* group (median 8, range 3–23; *post hoc* $P < 0.001$), the number of deletions per genome was higher in the *BRCA1* group (median 12, range 6–24) than in the sporadic group (median 9, range 3–17; *post hoc* $P < 0.01$). In addition, 31 previously unknown CNV regions were present specifically in the *BRCA1* group. When we performed pathway analysis on the 241 overlapping genes mapped to these novel CNV regions, the 'purine metabolism' and '14-3-3-mediated signaling' pathways were over-represented (Fisher's exact test, $P < 0.01$). Our study shows that there are qualitative differences in genomic CNV profiles between *BRCA1*-associated and sporadic ovarian cancer patients. Further studies are necessary to clarify the significance of the genomic CNV profile unique to *BRCA1*-associated ovarian cancer patients.

INTRODUCTION

A copy number variation (CNV) is a segment of DNA 1 kb or larger that is present at variable copy numbers in comparison to a reference genome (Feuk et al., 2006). Recently, germline CNVs have been recognized not only as causes of rare genetic disorders but also as important susceptibility factors for a range of common diseases including infectious, autoimmune, and neuropsychiatric diseases (Cohen, 2007; Wain et al., 2009; Zhang et al., 2009; Fanciulli et al., 2010). In the field of cancer research, somatic CNVs are detected in the genomes of various cancer cells, and some of them are thought to play an important role in carcinogenesis (Shlien and Malkin, 2009; Fanciulli et al., 2010). Shlien et al. (2008) reported excessive genomic CNVs in Li-Fraumeni syndrome (LFS), which is an autosomal dominant disorder characterized by increased risk of various cancers in individuals with germline *TP53* mutations. However, the role of germline CNVs in individuals predisposed to cancer or in cancer patients has not yet been clarified.

Familial ovarian cancer accounts for 5%–10% of all epithelial ovarian cancers (Whittemore et al., 1997), and most familial cases are associated with mutations in Breast cancer 1, early onset (*BRCA1*; Easton et al., 1995). *BRCA1* is located on chromosome 17q21 and plays critical roles in DNA repair, cell cycle checkpoint control, and maintenance of genomic stability (Gudmundsdottir and Ashworth, 2006). Histologically, most *BRCA1*-associated ovarian cancers are serous papillary adenocarcinomas (Sekine et al., 2001). Interestingly, the genomic profile for *BRCA1*-associated ovarian cancer is distinct from that for sporadic ovarian cancer, although they have the same histological type (Patael-Karasik et al., 2000; Zweemer et al., 2001; Israeli et al., 2003; Walsh et al., 2008). Therefore, it is thought that *BRCA1*-

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associated and sporadic serous ovarian cancers arise from different molecular pathways (Walsh et al., 2008). To date, studies regarding differences in germline CNVs between *BRCA1*-associated and sporadic ovarian cancers have been not reported.

Previously, we analyzed genetic alterations in *BRCA1* among Japanese ovarian cancer families, demonstrating the clinicopathological characteristics of *BRCA1*-associated ovarian cancer patients. In the present study, we sought to identify the genome-wide profile of germline CNVs in *BRCA1*-associated ovarian cancers using an ultra-high-resolution single nucleotide polymorphism (SNP) array and to elucidate the molecular characteristics of germline CNVs in *BRCA1*-associated ovarian cancer patients, when compared to sporadic ovarian cancer patients or healthy women.

MATERIALS AND METHODS

Subjects

In this study, we recruited 71 individuals with germline *BRCA1* mutations, 47 healthy controls aged >65 years with no history of cancer, and 34 sporadic ovarian cancer patients without any familial history of cancer. Patients and controls were all of Japanese ethnicity. The ethics committees of the participating institutions approved the study protocol and each participant gave written informed consent. Germline mutations in *BRCA1* were examined according to an in-house protocol (Sekine et al., 2001). Genomic DNA was prepared from lymphocytes using the standard phenol-chloroform method.

SNP Array Experiments

For analysis with the Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA), genomic DNA preparation and chip processing were performed according to the Affymetrix recommended protocols. Briefly, 250 ng of genomic DNA was digested with either *Nsp*I or *Sty*I (New England Biolabs, Inc., Ipswich, MA) and then ligated to Nsp or Sty adaptors, respectively. The adaptor-ligated DNA fragments were amplified by polymerase chain reaction (PCR) using a single primer that recognized the adaptor sequence. All products from each sample were combined and purified using magnetic beads (Agencourt AMPure, Beckman Coulter, Beverly, MA). The purified PCR products were fragmented using DNase I, end-labeled with a biotinylated nucleotide, and hybridized to a Genome-Wide Human SNP Array 6.0 (Affymetrix) at 50°C for 17 hr. After hybridization, the arrays were washed, stained, and scanned with a GeneChip Scanner 3000 7G (Affymetrix).

Data Analysis

Quality control (QC) was performed using Genotyping Console 4.0 (Affymetrix). The median of the absolute values of all pairwise differences between $\log_2$ ratios for a given chip (MAPD) was calculated for all samples. Samples with a MAPD value greater than 0.30 were not included in the study. The QC call rate of recruited samples was higher than 90%. After sample QC, chip data from 68 *BRCA1* mutation carriers (51 affected carriers and 17 carriers without cancer), 47 healthy controls, and 33 sporadic ovarian cancer patients were used in the subsequent analysis.

We prepared a custom reference panel for array-based CNV analysis. Although 270 HapMap samples have been used in many reports for copy number analysis (Redon et al., 2006; McCarroll et al., 2008), the 270 HapMap samples are from multiethnic populations and derived from lymphoblastoid cell lines. Previously, it was shown that there are variations in germline CNV distribution among different ethnic populations (White et al., 2007; Jakobsson et al., 2008; Li et al., 2009). Therefore, we selected SNP Array 6.0 data obtained from 330 Japanese women (Adachi et al., in press) as a reference, based on Affymetrix recommendations.

Data were processed using Partek Genomics Suite 6.5 (Partek Inc., St. Louis, MO). Genotype calls for the SNP probes were determined using the Birdseed v2 algorithm (Korn et al., 2008; Nishida et al., 2008). SNP call rates were higher than 97% in all samples. Regions of copy number alterations were detected using a Hidden Markov Model algorithm with the following parameters: maximum probability = 0.98, genomic decay = 1,000,000, and sigma = 1 (Walter et al., 2009). Because the median intermarker distance for the Genome-Wide Human SNP Array 6.0 is 680 bp, a CNV was defined as a change in the inferred copy number state in a genomic region covering at least five consecutive probes to avoid detecting false positive CNVs (Pinto et al., 2010). Gene annotation and overlap was determined using the University of California, Santa Cruz (UCSC) genome assembly (hg18) (http://genome.ucsc.edu/).

Detected CNVs in *BRCA1*-associated sporadic ovarian cancer patients and healthy controls were evaluated in terms of frequency and length. When we compared the frequency or length of CNV regions among *BRCA1*-associated sporadic ovarian cancer patients and healthy controls, the Kruskal-Wallis test was performed using GraphPad PRISM version 4.0 (GraphPad Software, San Diego, California, USA). When the Kruskal-Wallis test showed a significant difference, Dunn's multiple comparison test was performed as a *post hoc* test. *Post hoc* $P < 0.05$ was considered statistically significant. In addition, we used the Spearman rank correlation to measure the association between the number and length of CNVs per individual or between the number of CNVs and onset age at diagnosis using GraphPad PRISM version 4.0.

Genome CNV profiles measured by SNP Array 6.0 were compared between *BRCA1*-associated and sporadic ovarian cancer patients. Based on genome-wide CNV data for individuals, hierarchical clustering analysis was performed using a complete linkage clustering algorithm (Partek Genomic Suite 6.5) with Pearson correlation coefficients in all pairwise combinations (Haverty et al., 2009).

To investigate the biological characteristics of overlapping genes in detected CNV regions, Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com/) was used (Cuscó et al., 2009; Yoshihara et al., 2010). We performed 'Core Analysis' to examine which molecular function categories or pathways were statistically over-represented among the obtained gene list. Statistical significance was determined by Fisher's exact test using all annotated genes in IPA as a background.

RESULTS

Germline *BRCA1* Mutations

In this study, we evaluated 68 *BRCA1* mutation carriers in 41 *BRCA1* families. Of the 68 carriers, 51 were affected with ovarian cancer, and the median age at diagnosis was 52 years (range, 28–74 years). Histological analysis of the 51 patients with ovarian cancer showed that 43 (84.3%) were of the serous type, 6 (11.8%) were of the endometrioid type, and 2 (3.9%) were undifferentiated. The types of germline mutations in *BRCA1* are listed in Supporting Information Table 1. Nineteen *BRCA1* families were assigned to carriers with two types of nonsense mutations, L63X and Q934X, both of which are mutations specific to Japanese ovarian cancer patients (Sekine et al., 2001).

Identification of Germline CNVs

Germline CNVs in all samples were analyzed according to both signal intensity and genotyping data using the Affymetrix Genome-Wide Human SNP Array 6.0. To clarify the molecular characteristics of germline CNVs in *BRCA1*-associated ovarian cancer patients, we compared genome-wide profiles of germline CNVs among 51 *BRCA1* mutation carriers affected with ovarian cancer (*BRCA1* group), 33 sporadic ovarian cancer patients (sporadic group), and 47 healthy controls (normal group). The median ages were 52 years (range, 28–74) in the *BRCA1* group, 59 years (range, 34–81) in the sporadic group, and 73 years (range, 65–83) in the normal group.

Figure 1 shows a genome-wide frequency plot of germline CNVs in each group. Recurrent genomic changes among samples were observed at several chromosomal intervals. Most notably, CNVs at 1q21.3, 2q22.3, 4p16.1, 7q34, and 15q11.2 were observed in greater than 40% of each group. These CNVs had been previously reported in more than ten publications and submitted to the Database of Genomic Variants (http://projects.tcag.ca/variation/).

To evaluate the differences in CNV frequencies between the three groups, a Kruskal-Wallis test was performed. The total number of CNVs per genome was higher in the sporadic group (median 26, range 12–34) than in the *BRCA1* (median 21, range 11–35; *post hoc* $P < 0.05$) or normal group (median 20, range 7–32; *post hoc* $P < 0.05$; Fig. 2). Regarding the number of amplification regions, there were significant differences between the sporadic group (median 13, range 7–26) and the other two groups (*BRCA1* group: median 8, range 3–23; *post hoc* $P < 0.001$; normal group: median 9, range 1–20; *post hoc* $P < 0.001$). On the other hand, the number of deletion regions in the *BRCA1* group was higher than in the sporadic group (*post hoc* $P < 0.01$). The deletion/amplification ratio, which is, the per-sample ratio of the number of deletion regions to the number of amplification regions, yielded a median of 1.50 (range 0.50–7.00) in the *BRCA1* group, 1.30 (range 0.40–6.00) in the normal group, and 0.60 (range 0.30–1.70) in the sporadic group. In addition, we analyzed the differences in the lengths of CNV regions per individual among the three groups. Although the lengths of total CNVs per genome or amplification regions were

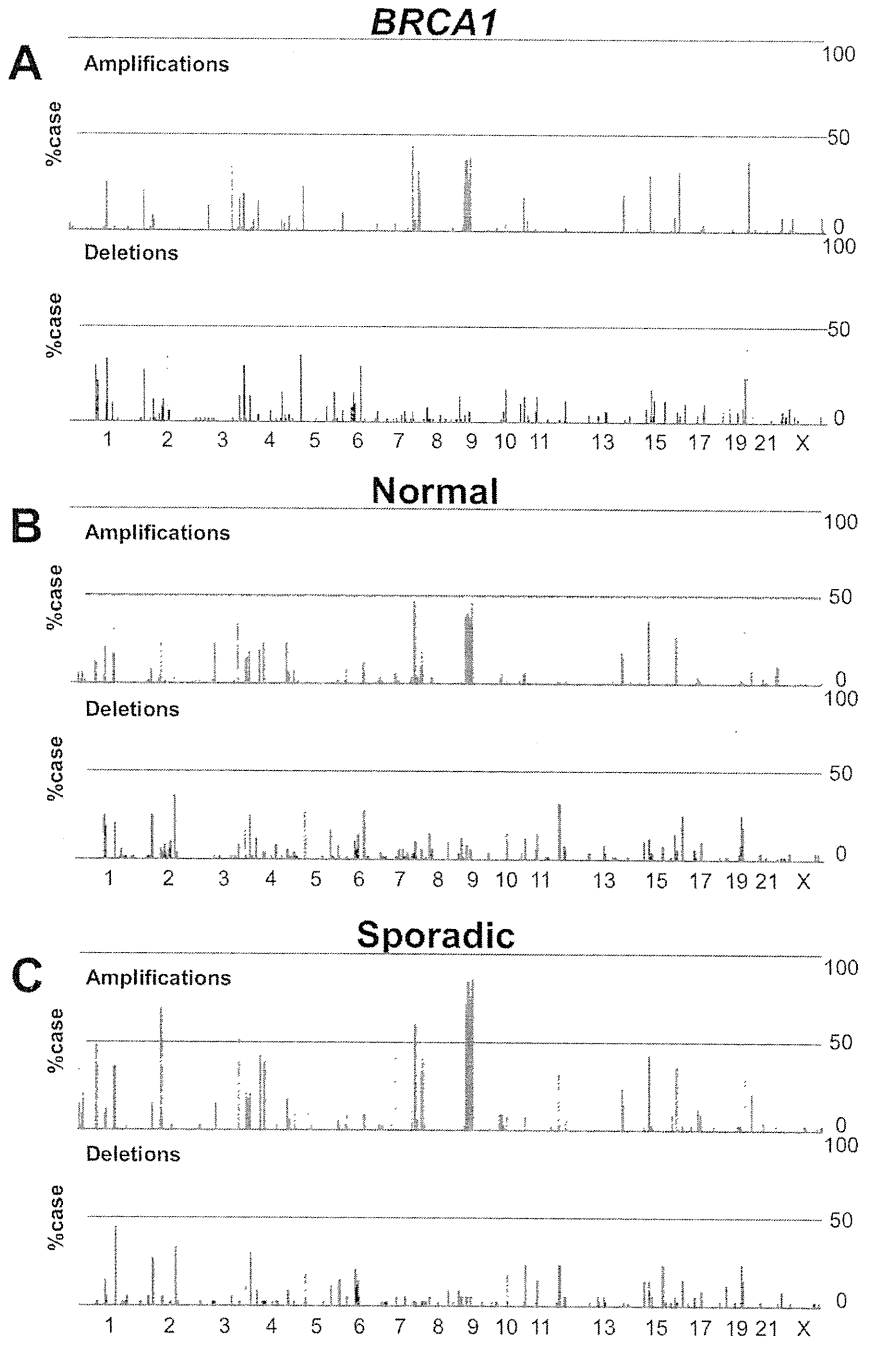

Figure 1. Frequency plot summarizing germline CNVs in *BRCA1*, sporadic, and normal groups. Amplifications are shown in red and deletions in blue. The genomic location is represented along the *x*-axis, with chromosome 1 on the left and the X chromosome on the right. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significantly greater in the sporadic group than in the *BRCA1* (total CNV, *post hoc* $P < 0.001$; amplification, *post hoc* $P < 0.001$) or normal groups (total CNV, *post hoc* $P < 0.001$; amplification, *post hoc* $P < 0.001$), there was no difference in the total length of the deletion regions among the three groups (Kruskal-Wallis test, $P = 0.55$; Supporting Information Fig. 1). Spearman's correlation analysis for all samples indicated that there was a significant correlation between the total number and total length of CNV regions (r: 0.47, $P < 0.0001$).

We focused our attention on small-scale CNVs, defined as segment sizes less than 50 kb, which were detected by new ultra-high-resolution techniques, because these small-scale CNVs could

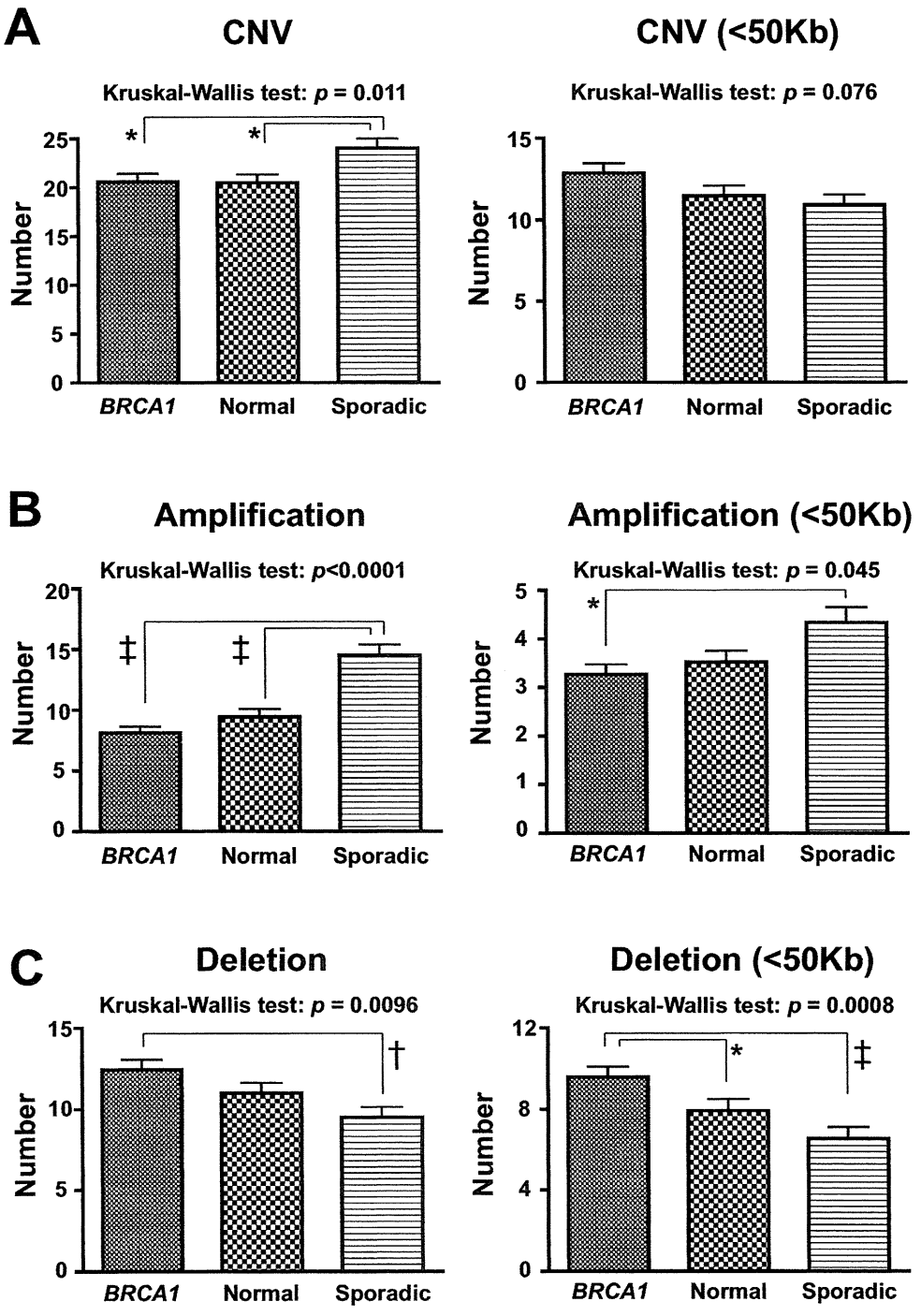

Figure 2. Differences in CNV frequency among *BRCA1*, sporadic, and normal groups. There are significant differences in total CNV number (A), amplification (B), and deletion regions (C) among the three groups. The cutoffs for *post hoc P* values in Dunn's multiple comparison test were set to 0.05 (*), 0.01 (†), and 0.001 (‡). When small-scale CNVs are defined as less than 50 kb, the results for the number of small-scale CNVs (A), amplification (B), and deletion regions are indicated in the right column.

not have been analyzed by previous methods such as clone-based array comparative genome hybridization or fluorescence *in situ* hybridization (Tuzun et al., 2005; Feuk et al., 2006). Interestingly, the number of small CNV regions in the *BRCA1* group was marginally higher than in the other two groups ($P = 0.076$). The *BRCA1* group had higher frequencies of small deletion regions than the other groups (sporadic, *post hoc* $P < 0.001$; normal, *post hoc* $P < 0.05$; Fig. 2).

Because each group displayed several features concerning the frequencies or lengths of CNV regions per individual, we next assessed whether there were differences in genomic CNV profiles between the three groups. We performed hierarchical clustering analysis using genome-wide CNV data for individuals. In this clustering analysis, we included 17 *BRCA1* mutation carriers who did not have ovarian cancer to investigate differences in genomic CNV profiles between carriers affected and not affected with ovarian cancer. However, we could not identify any clear clustering patterns that divided each group. Hierarchical clustering analysis could not discriminate between affected and unaffected carriers (Supporting Information Fig. 2). Indeed, there were no significant differences in CNV frequencies between 51 *BRCA1* mutation carriers affected with ovarian cancer and 17 unaffected carriers (data not shown).

It is known that there is inter-individual variability in the age of onset for ovarian cancer in *BRCA1* patients (Sekine et al., 2001; Sugano et al., 2008). Our study showed no significant linear correlation between age at diagnosis and CNV frequency (data not shown). When we compared five *BRCA1* carriers afflicted with ovarian cancer before age 40 (early-onset) to six *BRCA1* carriers not affected before age 65 (including three cases afflicted with ovarian cancer after age 65), there was no significant difference in CNV frequency between the two groups (data not shown).

BRCA1-Unique CNVs

To clarify the qualitative differences in genomic CNV profiles between *BRCA1*-associated and sporadic ovarian cancer patients, we investigated unique CNVs detected only in the *BRCA1* group compared to the normal and sporadic groups. In total, 111 CNVs were detected only in the *BRCA1* group. Furthermore, we selected novel CNVs that had not been registered in the Database of Genomic Variants (http://projects.tcag.ca/variation) as *BRCA1*-unique CNVs, according to the criteria that two CNVs are the same when they overlap for more than 70% of their predicted genomic regions (Cuscó et al., 2009). As a result, we found 31 *BRCA1*-unique CNV regions, all of which include at least one gene (Table 1). Of these 31 CNV regions, 3 CNVs on 1q43, Xp22.2-21.1, and Xp21.3 were shared among ovarian cancer patients (sisters or mother and daughter) from the same family. However, because samples from both parents of *BRCA1* mutation carriers were not available, we could not evaluate whether other CNV regions arose *de novo* or were the result of inherited genomic changes. In a similar way, 25 and 13 regions were detected as sporadic ovarian cancer-unique CNVs and normal-unique CNVs, respectively (Supporting Information Tables 2 and 3).

To elucidate the biological characteristics of the overlapping genes included in *BRCA1*-unique or sporadic-unique CNV regions, we used the 'Core Analysis' embedded in IPA software. Because we had excluded miscRNAs or pseudogenes from this analysis, 241 *BRCA1*-unique, 129 sporadic-unique, and 37 normal-unique overlapping genes were uploaded to the IPA software. To characterize the gene list based on IPA classification of molecular and cellular functions, we examined which predefined categories were highly associated with group-unique overlapping genes using Fisher's exact test. When the P value cutoff was set to 0.001 for the purpose of clarifying differences among the three groups, three categories ('cellular development', 'cellular movement', and 'small molecular biochemistry') were over-represented in the *BRCA1* group (P = 0.00032, 0.00036, and 0.00079, respectively), and the 'cellular development' category was over-represented in the sporadic group (P = 0.00056; Supporting Information Table 4). Genes involved in 'cellular development' were notably enriched in both *BRCA1*- and sporadic-unique genes. On the other hand, there was no category associated with overlapping genes in normal-unique CNVs.

When we further investigated the association between overlapping genes in the *BRCA1*-unique CNVs and 'ingenuity canonical pathways' excluding 'disease-specific pathways', the 'purine metabolism' and '14-3-3-mediated signaling' pathways were significantly over-represented (Fisher's exact test, $P < 0.01$). Of 241 genes in *BRCA1*-unique CNVs, nine genes (*ENTPD8*, *ABCA2*, *GUCY1A3*, *ATP5D*, *ENTPD2*, *POLR2E*, *POLRMT*, *APRT*, *POLR2K*) were mapped to the 'purine metabolism' pathway that contains 439 genes, and five genes (*TRAF2*, *TP73*, *TUBB2C*, *PLCH2*, *PRKCZ*) were mapped to the '14-3-3-mediated signaling' pathway that contains 116 genes. In contrast, we could not identify any significant association between 'Ingenuity canonical pathways' and overlapping genes in sporadic ovarian cancer or normal-unique CNVs.

DISCUSSION

We investigated quantitative and qualitative differences in germline CNVs between *BRCA1*-associated and sporadic ovarian cancer patients.

TABLE 1. List of CNVs Unique to *BRCA1*-Associated Ovarian Cancer Group

Cytoband	Start	Length (Kb)	Genomic markers	CNV type	*BRCA1* ovarian cancer patients[a] (families)	Overlapping genes
1p36.33–32	1886992	1080.9	321	Amplification	1[b]	*ACTRT2, C1orf86, C1orf93, GABRD, HES5, LOC100128003, LOC100129534, LOC115110, MMEL1, MORN1, PANK4, PEX10, PLCH2, PRKCZ, RER1, SKI, TNFRSF14, FLJ42875, KIAA1751*
1p36.32	2967886	666.3	307	Amplification	2 (2)[b]	*ARHGEF16, FLJ42875, MEGF6, MIR551A, PRDM16, TPRG1L, WDR8, FLJ42875, TP73*
1p36.32	3634218	41.5	27	Amplification	1[b]	*KIAA0495, TP73, CCDC27*
1p22.2	88867379	216.4	118	Amplification	1	*PKN2*
1q12	131939195	10981.6	45	Amplification	1	*FLJ39739, LOC100130000, LOC100286793, PPIAL4G*
1q21.1	142920843	767.0	108	Amplification	1	*C1orf152, LOC728855, LOC728875, NBPF9, PDE4DIP, PPIAL4A, PPIAL4B, PPIAL4C*
1q25.3	182456954	176.5	140	Amplification	1	*C1orf21*
1q43	235562021	1044.5	874	Amplification	2 (1)	*LOC100130331, ZP4, RYR2*
3p14.1	67590604	133.2	81	Deletion	1	*SUCLG2*
4p14	39012069	167.5	76	Amplification	1	*KLB, LIAS, LOC401127, RPL9, UGDH, RFC1*
4p13	42600475	516.6	364	Amplification	1	*GRXCR1*
4q32.1	156809227	23.3	45	Deletion	1	*GUCY1A3*
6q26	162272312	128.2	113	Deletion	1	*PARK2*
7q31.32	122386377	329.1	254	Amplification	1	*SLC13A1, TAS2R16*
7q36.1	149174693	7.5	6	Amplification	1[b]	*ZNF862*
8q12.3	63510906	58.0	44	Deletion	2 (2)[b]	*NKAIN3*
8q22.2	101181884	277.9	135	Deletion	1[b]	*FBXO43, POLR2K, RNF19A, SPAG1, RGS22*
8q24.3	143194942	708.6	290	Amplification	1[b]	*ARC, BAI1, C8orf55, JRK, LY6D, LY6K, LYNX1, LYPD2, NCRNA00051, PSCA, SLURP1, TSNARE1*
9p21.2	28036146	7.0	5	Deletion	1[b]	*LINGO2*
9p13.1	38352179	417.5	390	Amplification	1	*ALDH1B1, ANKRD18A, C9orf122, IGFBPL1*
9p34.3	138083118	1539.2	421	Amplification	1	*ABCA2, AGPAT2, ANAPC2, C8G, C9orf139, C9orf140, C9orf142, C9orf163, C9orf167, C9orf169, C9orf172, C9orf173, C9orf69, C9orf75, C9orf86, CARD9, CLIC3, COBRA1, DNLZ, DPP7, EDF1, EGFL7, ENTPD2, ENTPD8, EXD3, FAM166A, FAM69B, FBXW5, FUT7, GPSM1, GRIN1, INPP5E, KIAA1984, LCN10, LCN12, LCN15, LCN6, LCN8, LCNL1, LHX3, LOC100131193, LOC100289341, LOC26102, LRRC26, MAMDC4, MAN1B1, MIR126, MRPL41, NDOR1, NELF, NOTCH1, NOXA1, NPDC1, NRARP, PHPT1, PMPCA, PNPLA7, PTGDS, QSOX2, RNF208, SDCCAG3, SEC16A, SLC34A3, SNAPC4, SNHG7, SNORA17, SNORA43, SSNA1, TMEM141, TMEM203, TRAF2, TUBB2C, UAP1L1, WDR85, ZMYND19, ARRDC1, NACC2*
10p11.23	30850068	137.7	138	Amplification	1	*LYZL2*
10q21.3	68086926	151.1	118	Deletion	1	*CTNNA3*
11p15.5	1852541	575.6	227	Amplification	1[b]	*ASCL2, C11orf21, CD81, H19, IGF2, IGF2AS, INS, INS-IGF2, LOC100133545, MIR483, MIR675, MRPL23, TH, TNNT3, TRPM5, TSPAN32, TSSC4, KCNQ1, LSP1*
15q24.2–3	86853024	953.6	327	Amplification	1[b]	*ACSF3, APRT, C16orf81, CBFA2T3, CDH15, CDT1, CTU2, CYBA, FAM38A, GALNS, IL17C, MGC23284, MVD, PABPN1L, RNF166, SNAI3, TRAPPC2L, ZC3H18, ZFPM1, ZNF469*
18q12.2	35402353	102.2	50	Deletion	1	*LOC647946*

(Continued)

TABLE 1. List of CNVs Unique to *BRCA1*-Associated Ovarian Cancer Group (Continued)

Cytoband	Start	Length (Kb)	Genomic markers	CNV type	*BRCA1* ovarian cancer patients[a] (families)	Overlapping genes
19p13.3	41910	1431.1	353	Amplification	1[b]	*ABCA7, ADAMTSL5, APC2, ARID3A, ATP5D, AZU1, BSG, C19orf20, C19orf21, C19orf22, C19orf23, C19orf24, C19orf25, C19orf26, C19orf6, C2CD4C, CDC34, CFD, CIRBP, CNN2, DAZAP1, EFNA2, ELANE, FGF22, FLJ45445, FSTL3, GAMT, GPX4, GRIN3B, GZMM, HCN2, HMHA1, KISS1R, LPPR3, MADCAM1, MED16, MIDN, MIER2, MUM1, NDUFS7, ODF3L2, OR4F17, PALM, PCSK4, POLR2E, POLRMT, PPAP2C, PRSSL1, PRTN3, PTBP1, REEP6, RNF126, RPS15, SBNO2, SHC2, STK11, THEG, WDR18*
19q13.41	58870566	149.4	108	Amplification	1[b]	*NLRP12*
19q13.43	61144359	1050.6	727	Amplification	1	*GALP, MIMT1, NLRP5, NLRP8, PEG3, PEG3AS, ZFP28, ZIM2, ZNF444, ZNF470, ZNF471, ZNF542, ZNF582, ZNF583, ZNF667, ZNF71, ZNF787, ZNF835, ZSCAN5A, ZSCAN5B*
Xp22.2–21.13	16903210	736.8	440	Amplification	4 (2)	*NHS, REPS2*
Xp21.3	28703508	109.2	100	Amplification	4 (2)	*IL1RAPL1*

[a]Numbers of *BRCA1* families were provided in the parenthesis.
[b]This means that this CNV was not detected in other member of the same family.

Our data show no significant difference in CNV frequency between *BRCA1*-associated ovarian cancer patients and healthy women (Fig. 2). Previous studies using low-resolution genetic analysis reported that allelic imbalance is increased threefold in normal breast epithelium from *BRCA1* mutation carriers, compared to controls (Larson et al., 2005; Clarke et al., 2006). Recently, Rennstam et al. (2010) used high-resolution array-based comparative genomic hybridization to show that genomic alterations in histopathologically normal breast tissue from *BRCA1* mutation carriers are more frequent than in the normal breast tissue of age-matched controls. In addition, it has been reported that different tissues vary in genomic copy numbers in the same individual (Piotrowski et al., 2008). Therefore, epithelial cells in breast or ovarian tissue might be more influenced than lymphocytes by *BRCA1* haploinsufficiency. In the future, analysis of genomic CNVs in both normal ovarian epithelium cells and lymphocytes from *BRCA1* mutation carriers who undergo prophylactic salpingo-oophorectomy will be required.

Deletions in primary lymphocyte DNAs from *BRCA1*-associated ovarian cancer patients were found more frequently than those from sporadic ovarian cancer patients or normal healthy women (Fig. 2). The use of an ultra-high-resolution SNP array allowed small-scale CNVs to be detected. The total number of small-scale deletions in the *BRCA1* group was also higher than in the other groups, as was the total number of deletions. These findings are consistent with Rennstam's result, where deletions were more frequently observed than amplifications in histopathologically normal breast tissues from *BRCA1* mutation carriers (Rennstam et al., 2010). It is thought that loss of homologous recombination by inactivation of BRCA1 results in inappropriate repair of double-strand DNA breaks via nonhomologous end-joining and single strand annealing, which leads to genomic instability through increased deletions or translocations (Moynahan et al., 1999; Venkitaraman, 2002; Turner et al., 2004; Walsh et al., 2008). Cousineau and Belmaaza (2007) indicated that breast cancer MCF7 cells with *BRCA1* haploinsufficiency display reduced efficiency of DNA double-strand break repair via homologous recombination. It is necessary to determine whether the above-described mechanisms in epithelial cells apply to other cell types such as lymphocytes. Shlien et al. (2008) reported that the CNV frequency in primary lymphocyte DNA is significantly increased in *TP53* mutation carriers compared to normal controls or the *TP53* wild-type group, likely owing to *TP53* haploinsufficiency, and proposed a model for CNV generation in tumorigenesis: an individual at risk of developing early-onset cancer such as LFS has an excess of CNVs and acquires more CNV regions in DNA from normal cells as time passes (Shilien and

Malkin, 2009). Thus, the predominance of deletions in the *BRCA1* group observed in our analysis implies that *BRCA1* haploinsufficiency might affect genome-wide CNV profiles of germline DNAs in *BRCA1*-associated ovarian cancer patients. However, we could not clarify this phenomenon in this study because blood samples for genomic DNAs were obtained only after diagnosis of ovarian cancer. In the future, longitudinal analysis of CNVs of normal DNA in the same samples should allow us to identify the presence of 'acquired CNVs' in cancer patients with *BRCA1* mutations.

Sporadic ovarian cancer patients had a higher CNV frequency than *BRCA1*-associated ovarian cancer patients or healthy controls (Fig. 2). Although we examined the relationship between age and CNV frequency in each sample, there were no significant correlations between age and CNV frequency in any group or in all samples (data not shown). When we divided the sporadic group into early-stage ($n = 9$) and advanced-stage cases ($n = 24$), no significant differences in CNV frequencies between the two subgroups were detected (Mann-Whitney test, $P = 0.86$). These data suggest that inherited CNVs, but not acquired CNVs in normal lymphocyte DNAs, might contribute largely to the higher number of CNVs in sporadic ovarian cancer patients. It is interesting to note that some sporadic-unique CNVs such as 4q13.2 and 12p13.31 are shared by two sporadic cases or more (Supporting Information Table 2). Diskin et al. (2009) reported that an inherited common CNV at 1q21.1 is associated with neuroblastoma, and that there is a previously unknown neuroblastoma breakpoint family gene within the CNV at 1q21.1 that is implicated in early tumorigenesis. To clarify the significance of the higher CNV frequency in sporadic ovarian cancer patients, it is essential to consider germline CNVs not only as rare CNVs but also as copy-number polymorphisms by performing a large-scale case-control association study.

We identified 31 *BRCA1*-unique CNV regions covering 241 overlapping genes associated with three molecular and cellular functions (Table 1 and Supporting Information Table 3). A subset of the 241 overlapping genes in *BRCA1*-unique CNVs were further involved in 'purine metabolism' and '14-3-3-mediated signaling'. Nucleotide metabolism is an important pathway related to carcinogenesis and is directly associated with DNA repair, whereas 14-3-3-mediated signaling is involved in cell cycle regulation and apoptosis. Burga et al. (2009) demonstrated that *BRCA1* haploinsufficiency leads to an increased ability of clonal growth and proliferation in primary mammary epithelial cells from *BRCA1* mutation carriers. Structural variations of genes with these molecular functions in *BRCA1*-unique CNV regions might contribute to the development of the unique biological characteristics in *BRCA1*-mutated cells. Further functional analysis is required to examine whether the overlapping genes can function as *BRCA1*-associated ovarian cancer-modifying genes.

Although several studies related to somatic CNVs of tumor DNAs previously reported that *BRCA1*-associated ovarian cancers show a higher frequency of somatic CNVs than sporadic ovarian cancers (Patael-Karasik et al., 2000; Zweemer et al., 2001; Israeli et al., 2003; Ramus et al., 2003; Walsh et al., 2008), differences in genetic CNV background between *BRCA1* and sporadic groups have received little discussion. This study shows that genomic profiles of CNV in *BRCA1* carriers are qualitatively distinct from those in sporadic ovarian cancer patients. The present finding may provide a first step to evaluate the association of germline *BRCA1* mutations with genomic CNV profiles in ovarian cancer patients.

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ORIGINAL ARTICLE *Inhibitors*

Clinical pharmacological study of a plasma-derived factor VIIa and factor X mixture (MC710) in haemophilia patients with inhibitors – Phase I trial

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Summary. MC710, a combined product of plasma-derived activated factor VII (FVIIa) and factor X (FX) at a protein weight ratio of 1:10, is a novel bypassing agent for haemostasis in haemophilia patients with inhibitors. In this study, pharmacokinetic (PK), pharmacodynamic (PD) parameters and safety of single doses of MC710 were investigated in 11 male haemophilia patients with inhibitors in a non-bleeding state. This was a multi-centre, open-labelled, non-randomized, active controlled crossover, dose-escalation study of five doses (20–120 μg kg^{-1} of FVIIa) with re-administration of different MC710 dosages to the same subjects. The active controls were NovoSeven (120 μg kg^{-1}) and/or FEIBA (50 and 75 U kg^{-1}) which were used to compare PD parameters. The area under the curve (AUC) and maximum plasma concentration (C_{max}) of MC710 active ingredients increased dose-dependently within the range of 20 and 120 μg kg^{-1}. After administration of MC710, activated partial thromboplastin time (APTT) was dose-dependently improved and prothrombin time (PT) was shortened to approximately 6 s at 10 min, and APTT improvement and PT shortening effects were maintained until 12 h after administration of MC710 at all doses. No serious or severe adverse event was observed after administration of MC710; furthermore, several diagnostic marker values and those changes did not indicate any signs of disseminated intravascular coagulation (DIC). These results suggest that MC710 would have haemostatic potential equal to or greater than NovoSeven and FEIBA and was be tolerable when given at doses up to 120 μg kg^{-1}.

Keywords: factor VIIa, factor X, haemophilia, inhibitors, bypassing agents, PK/PD

Introduction

Bleeding in haemophilia patients with inhibitors is mainly controlled by bypassing agents, recombinant activated factor VII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Bagsværd, Denmark) and activated prothrombin complex concentrates (APCC; FEIBA®, Baxter International Inc., Deerfield, IL, USA), however; those agents cannot always provide complete haemostasis. Currently, an improved regimen including combination therapy of rFVIIa and APCC are proceeding on the development [1–3]. On the other hand, a rFVIIa analogue in which several amino acids are mutated has been developed to strengthen the haemostatic effect

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of rFVIIa [4] and a polyethylene glycol (PEG)-rFVIIa product with a long half-life is under development for prophylaxis [5]. Furthermore, the development of a recombinant factor IX (FIX) analogue activating factor X (FX) without coenzyme factor VIII (FVIII) is in progress [6].

MC710 is a new bypassing agent that is currently under development by Kaketsuken (Kumamoto, Japan) and which includes plasma-derived FVIIa and its substrate FX (mixing ratio by protein weight 1:10). In manufacturing MC710, three processes for viral elimination and inactivation (solvent/detergent, virus removal membrane and dry-heat process) are included [7]. A minute amount of activated FX (FXa) is produced in the filling process and after reconstitution of lyophilized product; however, it has been confirmed that the FXa is neutralized by the antithrombin (AT) added to the product [7].

The Km for the FVIIa-catalysed FX activation (0.16–0.25 μM) is higher than the FX concentration in plasma (approximately 8 μg mL^{-1}, 0.14 μM) in the presence or absence of tissue factor (TF), therefore, the increase in blood FX concentration is expected to enhance the efficacy of FVIIa-mediated FX activation [7,8]. In fact, an increase in the haemostatic effect by simultaneous administration of FVIIa and FX has been confirmed in various *in vitro* studies and in animal studies using a haemophilia B inhibitor monkey model and the bypassing effect of MC710 was also confirmed in a study using haemophilia inhibitor-like plasma [7,9]. The 1:10 ratio of FVIIa to FX in MC710 was decided to be optimal based on the results of several *in vitro* studies using haemophilia-like plasma and a reconstituted blood coagulation model. To be specific, in plasma with a FVIIa concentration of 1.0–1.5 μg mL^{-1} (obtained after intravenous rFVIIa administration at the standard doses of 90–120 μg kg^{-1} [10]) when FX is exogenously added 5–15 μg mL^{-1}, the remarkably poor coagulant activity in haemophilic plasma is improved to that when FVIII or FIX is added at 10–20% [7,9].

We conducted a clinical pharmacological study (Phase I trial) of MC710 single administration in haemophilia patients with inhibitors in a non-bleeding state to determine the pharmacokinetic (PK) parameters of FVIIa and FX, and to evaluate the pharmacodynamic (PD) parameters and the safety of MC710.

Materials and methods

Study design and investigation drugs

This was a multi-centre, open-labelled, non-randomized, active controlled crossover, clinical pharmacological study for Japanese male haemophilia patients with inhibitors. All subjects provided written informed consent by using the form approved by the institutional review board of each participating institute. This trial was performed by the MC710 trial group listed in Appendix. MC710 was intravenously administered at a single dose to the patients in a non-bleeding state using dose escalation (five doses) to evaluate the dose-dependency of the PK and PD parameters and to evaluate safety. Prior to the administration of MC710, NovoSeven and/or FEIBA were administered at a single clinical dose as an active self-control to patients with no haemorrhage. The dose of NovoSeven and FEIBA was set at 120 μg kg^{-1} and one usual clinical dose for each patient, 50 or 75 U kg^{-1}, respectively. Haemophilia B patients and haemophilia A patients with allergy or anamnestic response to FEIBA were permitted not to receive FEIBA. The MC710 dose was to be gradually increased from the initial dose of 20 μg kg^{-1} (hereinafter the dose of MC710 is expressed as the amount of FVIIa) to 40, 80, 100 and 120 μg kg^{-1}. In this study, we allowed re-administration of MC710 to the same subjects at the next dose level after an interval of 4 weeks or more. A safety committee was held prior to each increase of the MC710 dose and, after confirming that there were no safety problems, the dose was stepped up to the next level. The minimum sample size for each MC710 dosage was set at $n = 4$, which is the minimum number for statistical evaluation. The key inclusion and exclusion criteria for the subjects are shown in Table 1. Blood samples were taken as follows: prior to and 10 and 30 min, and 1, 2 and 6 h after administration in NovoSeven treated patients; prior to and 10 and 30 min, and 2, 6, 24 and 48 h after administration in FEIBA treated patients; and prior to and 10 and 30 min, and 1, 2, 6, 12, 24 and 48 h after administration in MC710 treated patients.

MC710 was supplied as a lyophilized product and was formulated with FVIIa 0.6 mg mL^{-1}, FX 6 mg mL^{-1}, AT 1.0 U mL^{-1}, human serum albumin 2.0% and other salts after reconstitution [7]. MC710, NovoSeven and FEIBA were provided by Kaketsuken, Novo Nordisk A/S and Baxter International Inc., respectively.

PK/PD assessments

The PK parameters of the MC710 active ingredients were determined measuring FVII clotting activity

Table 1. Key inclusion and exclusion criteria.

Key inclusion criteria
Patients: male congenital haemophilia A or B patients with inhibitors
Age: ≥16 years or <60 years
Inhibitor titer: ≥1.0 BU mL^{-1}
Key exclusion criteria
Patients with following symptoms
Hypercoagulability
History of DIC
Development of AIDS
Hypersensitivity to NovoSeven, FEIBA and other plasma products
Decompensated cirrhosis
Heart failure, angina or pathologic arrhythmia such as atrial fibrillation
Anaemia

(FVII:C), FX clotting activity (FX:C), FVII antigen (FVII:Ag) and FX antigen (FX:Ag) and analysed using a non-compartmental model with WinNonlin® (ver 5.1; Pharsight, Mountain View, CA, USA) software using baseline adjusted values (differences from values before administration). Recovery was calculated as the percentage of the dose in the plasma 10 min after administration. In the PD parameter analysis, activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin–antithrombin complex (TAT) and prothrombin fragment F_{1+2} (F1+2) were determined.

Safety assessments

For the safety assessments, subjective symptoms and objective findings were observed and an electrocardiogram, vital signs tests and a laboratory test were performed before and after MC710 administration. For the evaluation of disseminated intravascular coagulation (DIC), platelet counts, fibrinogen and D-dimer were measured immediately after MC710 administration. The observation period for adverse events of NovoSeven, FEIBA and MC710 was 6 h, 48 h and 4 weeks after the administration, respectively. Twelve weeks after the administration of MC710, the virologic tests and the serologic tests were conducted to detect the production of new viral antigens or antibodies.

Statistical analysis

The MC710 dose-dependency of PK and PD parameters was analysed using a mixed effects model with subject as a random effect and dose (including observation time in the PD parameter analysis) as a fixed effect. The difference in PD parameters between the MC710 and the active control was analysed using a mixed effects model with subject as a random effect and treatment and observation time as fixed effects. For those statistical analyses, SAS Release 9.1 (SAS Institute Inc., Cary, NC, USA) was used. All reported *P*-values are two-tailed and not adjusted for multiple testing. *P*-values <0.05 was considered to be statistically significant.

Results

Subjects

A total of 25 administrations of MC710 were given to 11 subjects (seven haemophilia A patients with inhibitors and four haemophilia B patients with inhibitors) at five dose rates after administrations of active controls (Fig. 1). The mean age of the 11 subjects was 27.2 years (17–41 years) and the mean body weight was 61.3 kg (46.5–86.2 kg). The FVIII and FIX inhibitor titres immediately before administration of the investigational

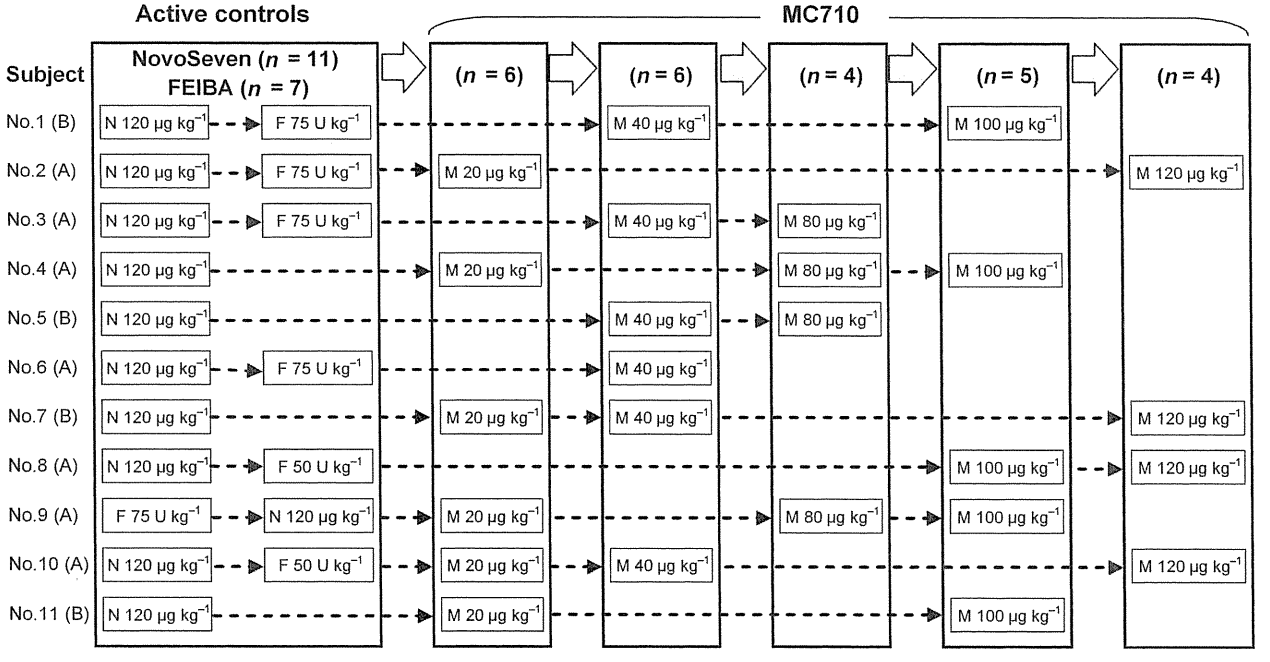

Fig. 1. Study flow chart. Prior to the administration of MC710, NovoSeven at 120 μg kg^{-1} and/or a dose of FEIBA at 50 or 75 U kg^{-1} were administrated as active controls. MC710 was administrated with dose escalation of 20, 40, 80, 100 and 120 μg kg^{-1}. The washout periods were 72 h, 2 weeks and 4 weeks after the administration of NovoSeven, FEIBA and MC710, respectively. The letter with the parenthesis after the subject No. indicates the type of haemophilia patient, with 'A' and 'B' indicating a haemophilia A patient with inhibitors, and a haemophilia B patient with inhibitors, respectively. M, N and F mean MC710, NovoSeven and FEIBA, respectively.

product ranged from 2.9 to 633 BU mL^{-1} and from 1.9 to 89.3 BU mL^{-1}, respectively.

Pharmacokinetics

Plasma FVII:C, FVII:Ag, FX:C and FX:Ag levels rapidly increased after administration of MC710. FVII:C and FVII:Ag levels returned to the preadministration values 12–24 h after administration (Fig. 2a and its inset, 2b and its inset) and increased levels of FX:C and FX:Ag persisted in the blood until 48 h after administration at MC710 doses of 80 μg kg^{-1} or more (Fig. 2c,d).

The results of the PK parameters showed MC710 dose-dependent increases in the area under the curve (AUC) and maximum plasma concentration (C_{max}) of FVII:C, FVII:Ag, FX:C and FX:Ag ($P < 0.001$). On the other hand, other parameters, half-life ($t_{1/2}$), mean residence time (MRT), clearance (CL), volume of distribution at steady state (Vd_{ss}) and recovery were not dependent on the MC710 dose (Table 2a–d).

Pharmacodynamics

APTT and PT. The APTT, prolonged 120 s or more before administration, was improved at a dose-dependent manner after administration of MC710 ($P = 0.01$), and the APTT improvement effect persisted until 12 h after administration at all doses (Fig. 3a). The APTT after administration of 100 and 120 μg kg^{-1} of MC710 was shorter than that after the administration of 120 μg kg^{-1} of NovoSeven (100 μg kg^{-1}: $P = 0.023$, 120 μg kg^{-1}: $P = 0.002$, and the APTT after administration of 80, 100 and 120 μg kg^{-1} of MC710 was shorter than that of 75 U kg^{-1} of FEIBA (80 μg kg^{-1}: $P = 0.049$, 100 μg kg^{-1}: $P = 0.022$ and 120 μg kg^{-1}: $P = 0.008$).

The PT reached approximately 6 s (the determination limit) after administration of all doses of MC710 except for 20 μg kg^{-1} and remained at that level for up to 2 h. At 6 h after administration of 80, 100 and 120 μg kg^{-1} of MC710, PT was shorter than that after the administration of 120 μg kg^{-1} of NovoSeven. PT reduction

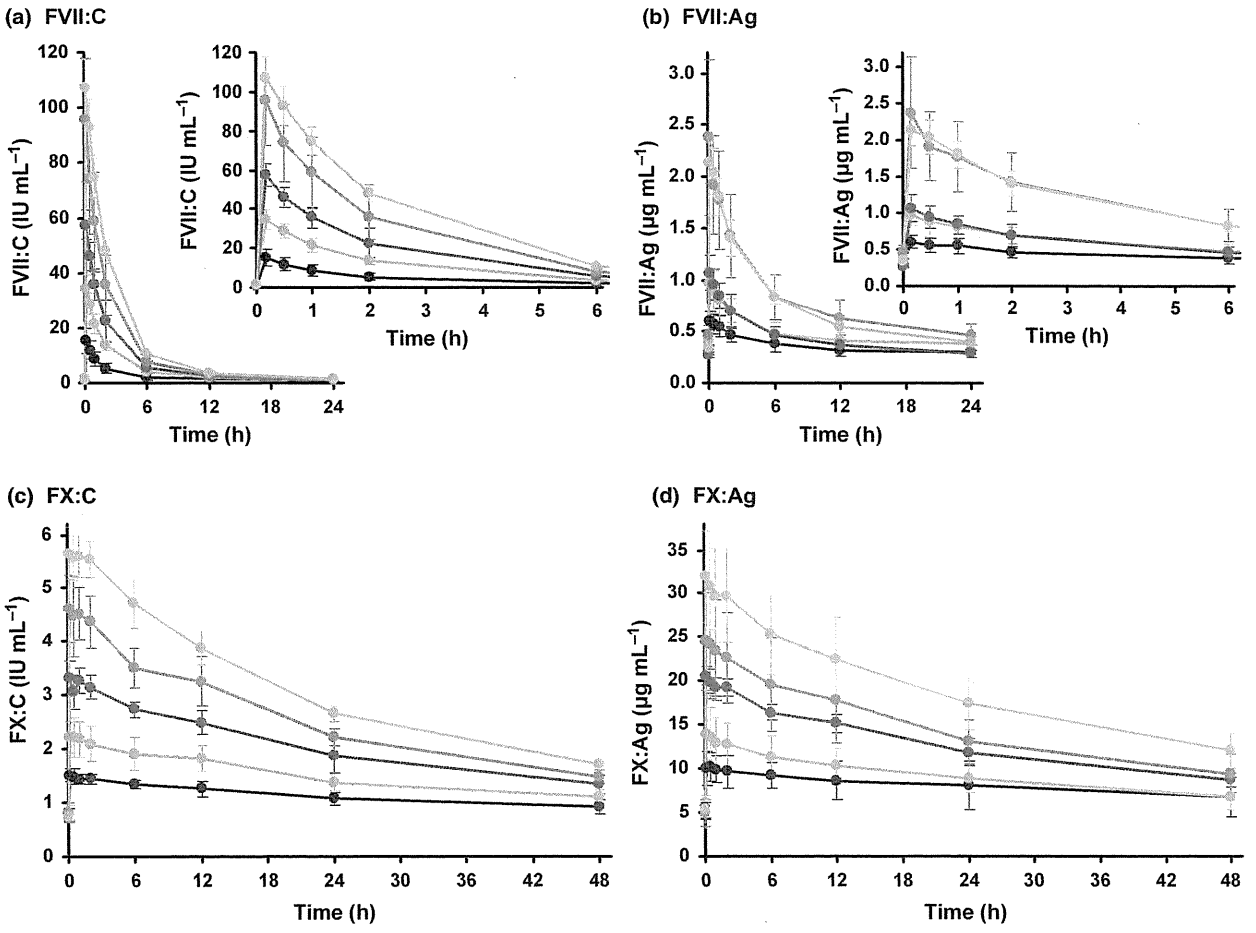

Fig. 2. Pharmacokinetics of MC710 ingredients. Time-dependent changes in the pharmacokinetics of FVII:C (a), FVII:Ag (b), FX:C (c) and FX:Ag (d) are shown. Enlarged figures of the changes in FVII:C and FVII:Ag until 6 h after administration are shown in the right upper corner of each graph. The mark represents the mean ± SD. MC710 doses are denoted as following colour symbols: 20 μg kg^{-1}, (—●—); 40 μg kg^{-1}, (—●—); 80 μg kg^{-1}, (—●—); 100 μg kg^{-1}, (—●—); 120 μg kg^{-1}, (—●—).

Table 2. PK parameters of MC710 ingredients.

(a) FVII:C

MC710 dose	*n*	$AUC_{0 \rightarrow 24}$ (IU h mL^{-1})	C_{max} (IU mL^{-1})	$t_{1/2}$ (h)	MRT (h)	CL (mL h^{-1} kg^{-1})	Vd_{ss} (mL kg^{-1})	Recovery (%)
20 μg kg^{-1}	6	31.2 ± 14.7	14.3 ± 4.2	2.1 ± 0.7	2.0 ± 0.7	37.2 ± 15.8	73.5 ± 20.7	70.7 ± 20.7
40 μg kg^{-1}	6	84.5 ± 11.2	33.2 ± 4.7	3.0 ± 1.2	2.4 ± 0.5	23.5 ± 3.5	61.4 ± 13.4	78.0 ± 9.8
80 μg kg^{-1}	4	151.8 ± 42.0	56.5 ± 5.5	3.4 ± 0.7	2.7 ± 0.3	27.8 ± 7.8	75.6 ± 13.0	71.1 ± 6.2
100 μg kg^{-1}	5	228.1 ± 62.7	94.2 ± 22.4	2.3 ± 0.5	2.2 ± 0.2	23.2 ± 6.6	52.8 ± 14.3	90.7 ± 18.6
120 μg kg^{-1}	4	296.3 ± 14.2	106.0 ± 10.2	2.8 ± 0.6	2.1 ± 0.2	20.1 ± 1.0	50.9 ± 5.5	83.4 ± 7.9

(b) FVII:Ag

MC710 dose	*n*	$AUC_{0 \rightarrow 24}$ (μg h mL^{-1})	C_{max} (μg mL^{-1})	$t_{1/2}$ (h)	MRT (h)	CL (mL h^{-1} kg^{-1})	Vd_{ss} (mL kg^{-1})	Recovery (%)
20 μg kg^{-1}	6	0.9 ± 0.7	0.3 ± 0.1	3.5 ± 2.4	2.5 ± 2.2	21.5 ± 12.5	80.0 ± 26.8	68.9 ± 18.1
40 μg kg^{-1}	6	1.9 ± 0.5	0.6 ± 0.1	4.2 ± 4.1	3.4 ± 2.4	20.0 ± 5.7	101.0 ± 79.1	66.4 ± 9.7
80 μg kg^{-1}	4	3.4 ± 1.4	0.8 ± 0.2	3.7 ± 1.5	3.8 ± 1.3	25.3 ± 10.8	105.1 ± 19.7	50.0 ± 9.6
100 μg kg^{-1}	5	7.0 ± 2.8	1.9 ± 0.7	4.9 ± 2.9	3.5 ± 1.4	13.9 ± 4.8	73.0 ± 31.5	92.0 ± 29.0
120 μg kg^{-1}	4	8.9 ± 1.8	1.8 ± 0.2	4.7 ± 1.5	4.8 ± 1.4	12.8 ± 2.8	79.5 ± 21.1	70.4 ± 7.6

(c) FX:C

MC710 dose	*n*	$AUC_{0 \rightarrow 48}$ (IU h mL^{-1})	C_{max} (IU mL^{-1})	$t_{1/2}$ (h)	MRT (h)	CL (mL h^{-1} kg^{-1})	Vd_{ss} (mL kg^{-1})	Recovery (%)
20 μg kg^{-1}	6	16.2 ± 2.8	0.7 ± 0.1	23.2 ± 8.3	16.5 ± 2.1	1.6 ± 0.5	47.0 ± 9.0	112.6 ± 17.9
40 μg kg^{-1}	6	34.0 ± 5.4	1.5 ± 0.2	22.5 ± 4.8	16.8 ± 1.6	1.5 ± 0.3	45.8 ± 9.0	104.1 ± 12.1
80 μg kg^{-1}	4	57.4 ± 6.1	2.5 ± 0.1	22.0 ± 0.8	16.8 ± 0.3	1.8 ± 0.2	53.6 ± 6.1	98.7 ± 7.8
100 μg kg^{-1}	5	79.8 ± 9.5	3.9 ± 0.6	20.2 ± 2.2	15.9 ± 0.8	1.6 ± 0.2	45.1 ± 6.8	114.7 ± 13.6
120 μg kg^{-1}	4	111.3 ± 11.6	5.0 ± 0.5	22.7 ± 1.5	16.4 ± 0.5	1.3 ± 0.1	41.5 ± 4.6	120.9 ± 11.4

(d) FX:Ag

MC710 dose	*n*	$AUC_{0 \rightarrow 48}$ (μg h mL^{-1})	C_{max} (μg mL^{-1})	$t_{1/2}$ (h)	MRT (h)	CL (mL h^{-1} kg^{-1})	Vd_{ss} (mL kg^{-1})	Recovery (%)
20 μg kg^{-1}	6	119.8 ± 59.5	4.8 ± 0.5	24.5 ± 12.1	17.2 ± 4.3	1.5 ± 0.6	50.8 ± 18.2	109.4 ± 17.6
40 μg kg^{-1}	6	199.3 ± 40.9	9.6 ± 1.9	22.8 ± 8.7	17.1 ± 2.4	1.6 ± 0.5	48.3 ± 13.2	105.4 ± 15.6
80 μg kg^{-1}	4	354.4 ± 42.9	15.5 ± 0.6	24.8 ± 1.5	17.4 ± 0.6	1.7 ± 0.2	58.2 ± 6.1	96.6 ± 4.7
100 μg kg^{-1}	5	441.6 ± 100.0	20.1 ± 4.6	24.8 ± 2.7	17.0 ± 0.7	1.7 ± 0.4	60.0 ± 15.0	94.0 ± 16.9
120 μg kg^{-1}	4	636.6 ± 81.3	26.6 ± 4.0	27.5 ± 2.5	17.8 ± 0.6	1.3 ± 0.1	51.7 ± 8.3	104.9 ± 16.7

The PK parameters were analysed using a non-compartment model. The PK of FVII:C and FVII:Ag were analysed using the data until 24 h after administration and those of FX:C and FX:Ag using the data until 48 h after administration. Data are shown as the mean ± SD.

persisted until 12 h after administration at all doses (Fig. 3b). The PT after administration of 40, 80, 100 and 120 μg kg^{-1} of MC710 was shorter than that of 75 U kg^{-1} of FEIBA (40 μg kg^{-1}: $P = 0.019$, 80 μg kg^{-1}: $P = 0.006$, 100 μg kg^{-1}: $P = 0.006$ and 120 μg kg^{-1}: $P = 0.005$).

TAT and F1+2. The TAT level increased above the upper limit for healthy individuals (3.2 ng mL^{-1}) 2 h after administration in all subjects except for two subjects with 20 μg kg^{-1} MC710. The increase in the TAT level was not dependent on the MC710 dose; however, the TAT level at 2 h after administration of MC710 was higher in the higher doses (Fig. 4a-inset). As one subject had an extremely high TAT level (85.8 ng mL^{-1}) at 6 h after administration of MC710 100 μg kg^{-1}, the mean at that point was high (the TAT levels of the other four subjects ranged from 3.0 to 10.4 ng mL^{-1}) (Fig. 4a). The investigator commented that the unusual increase of TAT level was caused by inadequate blood drawing at that time point.

The F1+2 level showed peaks at 2 h at MC710 administrations (>80 μg kg^{-1}), exceeding the upper limit for healthy individuals (229 pM) (Fig. 4b-inset). The increase in F1+2 was dependent on the MC710 dose ($P = 0.018$). The increase in F1+2 after administration of MC710 120 μg kg^{-1} was higher than that after administration of NovoSeven 120 μg kg^{-1} ($P = 0.01$). A sharp increase in F1+2 was observed 10 min to 2 h after administration of FEIBA (Fig. 4b). It was confirmed that the FEIBA product contained F1+2 (data not shown), resulting in the remarkable increase of F1+2.

Safety

No serious or severe adverse event was observed within 4 weeks after administration of MC710 and no subject discontinued the study due to an adverse event. The incidences of adverse events were not dependent on the MC710 dose. Headache, abdominal pain and oral herpes (all in one subject) occurred within 4 weeks after administration of MC710 at a dose of 40 μg kg^{-1} and a causal relationship with MC710 could not be ruled out. No clinical symptoms or changes in laboratory tests (platelet count, fibrinogen, D-dimer) indicating the hypercoagulable state such as DIC were induced after administration of MC710 (data not shown). In addition, the results of virologic and serologic tests confirmed that no subject developed a new viral antigen

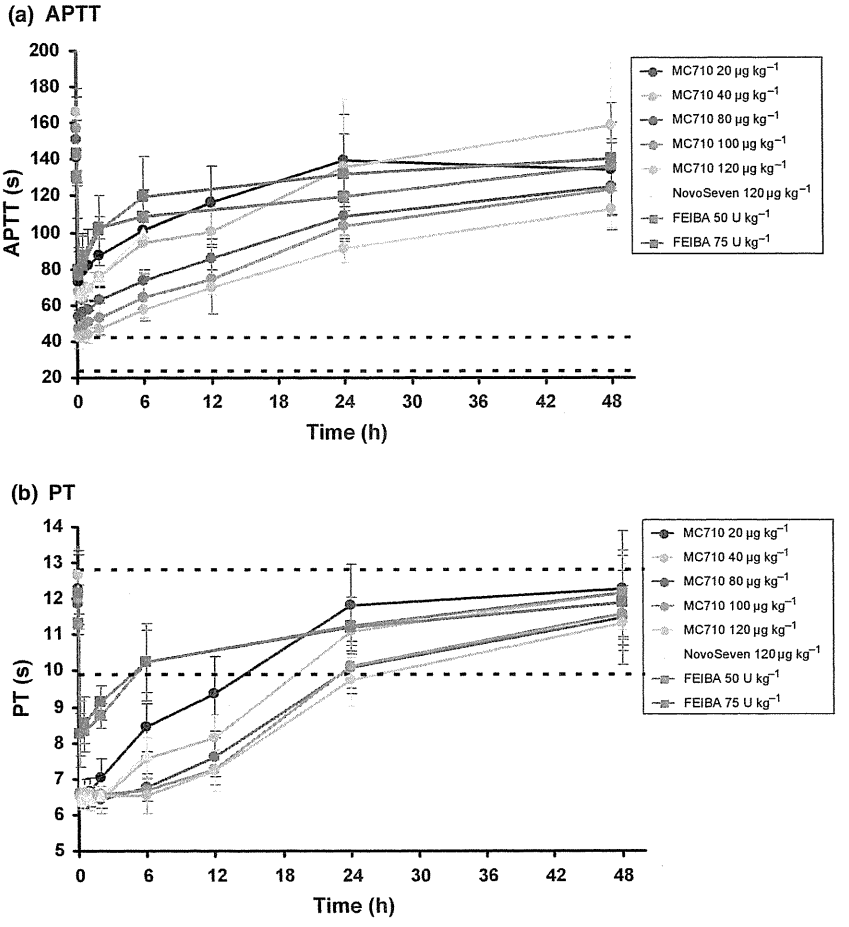

Fig. 3. Changes in APTT and PT. Time-dependent changes in APTT (a) and PT (b) are shown. The normal ranges for healthy individuals (...) for APTT were defined as 42.5 (upper limit) and 23.5 (lower limit) s and for PT as 12.8 (upper limit) and 9.9 (lower limit) s. The mark represents the mean ± SD.

or produced a new antibody after administration of MC710.

Discussion

The results of PK parameters showed that the AUC and C_{max} of FVII:C, FX:C, FVII:Ag and FX:Ag were dependent on the MC710 dose (Fig. 2a–d and Table 2). The mean $t_{1/2}$ of FVII:C in the MC710-treated ranged from 2.1 to 3.4 h (Table 2a) and was similar to the previous reported value (mean $t_{1/2}$ of FVII:C in haemophilia patients without haemorrhage: 2.82 ± 0.53 h [11]). On the other hand, the mean $t_{1/2}$ of FX:C ranged from 20.2 to 23.2 h (Table 2c) and was shorter than reported values ($t_{1/2}$ of FX:C 24–56 h [12,13]); however, this difference may have been due to the effect of the concurrent presence of FVIIa at a high concentration, different test conditions (agents, blood sampling point) and analytical method.

The results of the PD analysis also showed that MC710 had a strong ability to improve prolonged APTT and shorten PT in haemophilia patients with inhibitors. In fact, the mean APTT at 10 min after administration of 120 μg kg^{-1} of MC710 was substantially improved to the upper limit for healthy individuals (42.5 s) (Fig. 3a). The APTT improvement effect and PT shortening effect were consistent with the dose-dependent changes in the AUC and C_{max} of MC710 in the PK analysis. In comparison with the active control, from results on APTT and PT, it is suggested that MC710 at 40 μg kg^{-1} may have the haemostatic effect of equivalent to 120 μg kg^{-1} of NovoSeven. TAT and F1+2 was increased after administration of MC710 (Fig. 4a-inset and 4b-inset), indicating the results of prothrombin activation in blood flow; however, similar increases in TAT and F1+2 were also observed after the administration of NovoSeven and FEIBA (Fig. 4a,b) and several studies have also reported the increase in TAT [14,15].

When administration of MC710 increases the plasma FVIIa and FX concentration, FVIIa activates FX at a concentration similar to that in rFVIIa treatment on the activated platelet. An increased FX level in plasma would be beneficial for the amplification of the thrombin burst and would lead to a greater bypassing effect than that of FVIIa administration alone. On the other

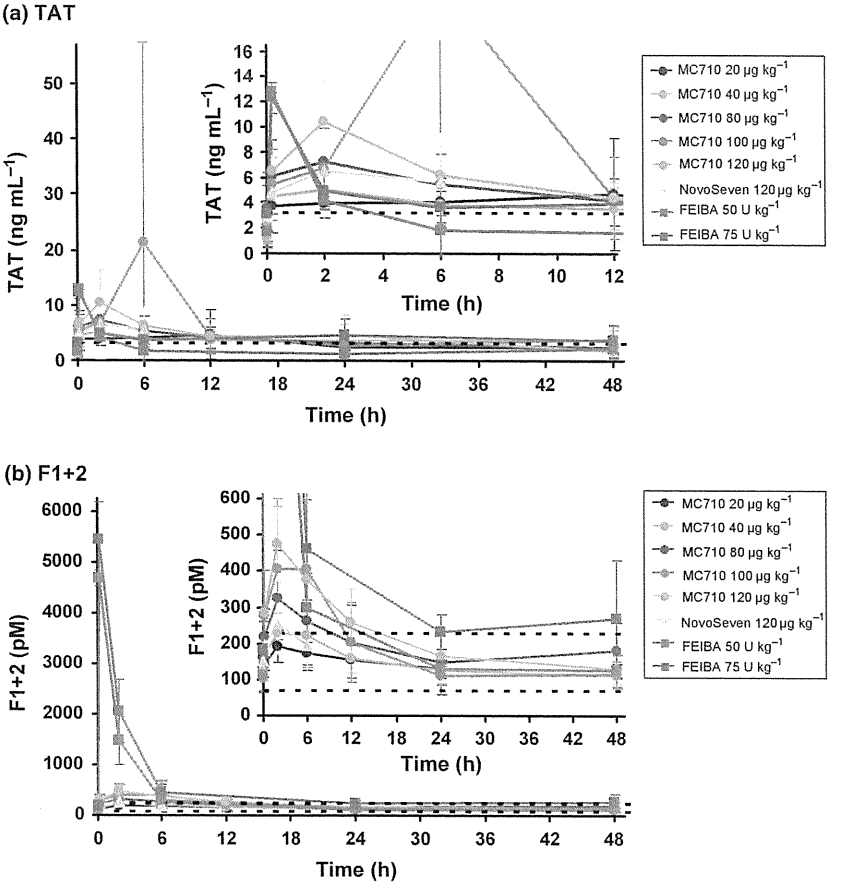

Fig. 4. Changes in TAT and F1+2. Time-dependent changes in TAT (a) and F1+2 (b) are shown. An enlarged figure of changes in TAT until 12 h after administration with an enlarged *Y*-axis is shown in the right upper corner of the graph. An enlarged figure of changes in F1+2 with an enlarged *Y*-axis is shown in the right upper corner of the graph. The normal range for healthy individuals (...) for TAT was defined as 3.2 ng mL^{-1} (upper limit, no lower limit) and for F1+2 as 229 (upper limit) and 69 (lower limit) pM. The mark represents the mean ± SD.

hand, the effects of MC710 on improving APTT and shortening PT persisted for 6–12 h after administration. These long acting effects may be attributed to the long half-life of FX ($t_{1/2}$ of FX:C: 20.2–23.2 h).

This study was non-randomized with a small sample size due to the fact that haemophilia patients with inhibitors are rare in Japan. However, by administration of active controls and re-administration of different doses of MC710 to the same subject, the inter-individual variability was reduced and internal validity was increased. Furthermore, a mixed effects model was applied for the statistical analysis to increase the sensitivity of analysis taking account of inter-individual variation.

In this study, PK and PD parameters changed in dose-dependent manners after administration of MC710 and the PD parameter changes were equal to or greater than those of the active controls. Furthermore, MC710 was safely administered at doses up to 120 μg kg^{-1} and no serious or severe adverse event, including DIC, was observed. These results suggest that MC710 has a dose-dependent haemostatic effect and the haemostatic effect is greater than that of the NovoSeven and FEIBA, and that MC710 has a tolerability of up to 120 μg kg^{-1}. An exploratory study (Phase II trial) in haemophilia patients with inhibitors who are haemorrhaging is planned to evaluate the efficacy and safety of MC710.

Addendum

Akira Shirahata developed the clinical trial protocol, evaluated efficacy and safety data and discussed the clinical events in the role of medical expert.

Hidehiko Saito, Katsuyuki Fukutake, Jun-ichi Mimaya, Junki Takamatsu and Midori Shima acted as the coordinating investigators, ensuring that investigators at the different institutions had a common understanding of the protocol and the implementation of the trial and gave general advice on the conduct of the trial.

The investigators had responsibility for all medical judgments associated with this trial in their institution and conducted the trial in accordance with the protocol including the selection of subjects, obtaining of informed consent, provision of data and information, reporting of adverse events, documentation of case reports and storage of essential documents.

Yasuo Ohashi, acting as the statistical advisor, gave advice and instruction on statistical analysis methods for the trial.

Kaketsuken managed the trial overall, including development and amendment of the protocol, data management, statistical analysis, quality control and assurance and data preservation.

Mitsubishi Chemical Medience Corporation collected the samples taken at the trial sites, conducted the tests, maintained measurement results and guaranteed the reliability of the results of the analyses.

Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan) was responsible for the estimation of PK parameters and conducted the statistical analysis.

Acknowledgements

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Disclosures

Akira Shirahata and Yasuo Ohashi received a fee from Kaketsuken for the implementation of the trial. Other authors have declared that no conflict of interest exists in this trial.

Appendix: MC710 Trial group

The institutions and the doctors participating in this trial are listed below.

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