J Hum Genet (2007) 52:856–864
DOI 10.1007/s10038-007-0183-9

ORIGINAL ARTICLE

High-resolution SNP and haplotype maps of the human gamma-glutamyl carboxylase gene (*GGCX*) and association study between polymorphisms in *GGCX* and the warfarin maintenance dose requirement of the Japanese population

Pei-Chieng Cha · Taisei Mushiroda · Atsushi Takahashi · Shigeru Saito · Hideki Shimomura · Takao Suzuki · Naoyuki Kamatani · Yusuke Nakamura

Received: 4 July 2007 / Accepted: 2 August 2007 / Published online: 5 September 2007

Abstract Gamma-glutamyl carboxylase (*GGCX*) plays an important role in blood coagulation through post-translational carboxylation of vitamin K-dependent blood-clotting proteins. This carboxylation process is impaired in the presence of warfarin, a vitamin K antagonist. Recent studies on *GGCX* have provided insights into association of polymorphisms in this gene, with inter-individual differences in the required warfarin maintenance dose. In order to provide a useful resource for further elucidating this association, we here report a high-resolution single nucleotide polymorphism (SNP) and haplotype maps of an 18-kb genomic region corresponding to the *GGCX* locus in the Japanese population. Among 41 SNPs, seven insertion/deletion polymorphisms, and a microsatellite polymorphism that we detected by direct sequencing of the DNAs of 96 Japanese individuals who were treated with warfarin, 32 genetic variations have not been reported. Using genotype information from 12 SNPs and the EM algorithm, we estimated haplotypes for this genomic region. Subsequently, we investigated associations of each of these polymorphisms with the warfarin maintenance-dose requirements of 828 Japanese patients, including the 96 patients that were used for DNA sequencing. We found no significant association between the polymorphisms in *GGCX* and the dose requirement.

Keywords Gamma-glutamyl carboxylase · Vitamin K-dependent proteins · Warfarin · Single nucleotide polymorphism · Inter-individual differences

Introduction

Human gamma-glutamyl carboxylase gene (*GGCX*), which is located on chromosome 2p12 (Kuo et al. 1995), spans about a 13-kb genomic region and consists of 15 exons (Wu et al. 1997). It encodes a 758-amino-acid protein that appears to have three transmembrane domains near its amino terminus (Wu et al. 1991). GGCX is also known as the vitamin K-dependent carboxylase, and activates a subset of proteins with calcium-binding properties, vitamin K-dependent (VKD) proteins or γ-carboxyglutamate-proteins (Gla-proteins), by catalysing them (Suttie 1985). Conversion from glutamyl residues to γ-carboxyglutamyl residues within VKD proteins allows VKD proteins to chelate calcium ions and subsequently undergo metal-dependent conformational alteration that is essential for these proteins to exert its biological functions (Furie and Furie 1988). Examples of VKD proteins are prothrombin, blood coagulation factors VII, IX, X, protein C, and protein S (FAO/WHO expert consultation on human vitamin and

Electronic supplementary material The online version of this article (doi:10.1007/s10038-007-0183-9) contains supplementary material, which is available to authorized users.

P.-C. Cha · Y. Nakamura (✉)
Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
e-mail: yusuke@ims.u-tokyo.ac.jp

T. Mushiroda · Y. Nakamura
Laboratory for Pharmacogenetics, RIKEN SNP Research Center, Tokyo, Japan

A. Takahashi · N. Kamatani
Laboratory of Statistical Analysis, RIKEN SNP Research Center, Tokyo, Japan

S. Saito · H. Shimomura · T. Suzuki
Tokushukai Hospital Group, Tokyo, Japan

mineral requirements 1998). These proteins require post-translational carboxylation before they can exert their functions in hemostasis (Furie and Furie 1990).

Previous studies demonstrated that post-translational carboxylation by GGCX is impaired in the presence of a vitamin K antagonist such as warfarin, an oral-anticoagulant, which creates the anticoagulant state by interfering with the production of reduced vitamin K, an essential cofactor for the carboxylation reaction (Esmon et al. 1975; Horton and Bushwick 1999). Although most of the variability observed in patients' response to warfarin may be attributed to genetic varations in *VKORC1* (Mushiroda et al. 2006; Schwartz and Stein 2006) that functions to regenerate reduced vitamin K (Rost et al. 2004; Li et al. 2004), polymorphisms in *GGCX* have also been indicated to have some association with inter-individual variation in warfarin maintenance-dose requirement (Shikata et al. 2004; Chen et al. 2005; Loebstein et al. 2005; Wadelius et al. 2005; Herman et al. 2006; Kimura et al. 2007; Vecsler et al. 2006).

In this study, we report identification of 41 SNPs, seven insertion/deletion polymorphisms (indels), and a microsatellite polymorphism in an 18-kb genomic region corresponding to human *GGCX* by direct sequencing of DNAs from 96 Japanese individuals, and also the haplotype structure of this region. We also evaluated the association between polymorphisms in *GGCX* and the warfarin maintenance-dose requirements of our Japanese subjects.

Materials and methods

Population samples

Eight hundred and twenty eight Japanese individuals under warfarin therapy were recruited for the current study. All individuals had given written informed consent to participate in the study in accordance with the process approved by the Ethical Committees of the Institute of Medical Science, University of Tokyo, Japan.

SNP discovery and genotyping methods

Initially, we carried out SNP discovery by using DNA samples of 96 of the 828 above-mentioned Japanese individuals; 32 individuals from each of three categories of patient (those requiring high, medium and low warfarin maintenance doses). The mean daily warfarin dose requirements of patients from each of these categories are 6.625, 3.5, and 0.95 mg respectively.

Using the genomic sequence information (GenBank accession number, AC016753.9), we designed 20 sets of primers (Supplementary Table 1) to amplify the 18-kb genomic region from 2.4 kb upstream of the first exon to 2.5 kb downstream of the last exon of *GGCX*, except for the repetitive sequences screened by the Repeat-Masker program (http://www.repeatmasker.org/). We defined exon–intron boundaries of *GGCX* by comparison of genomic sequences with cDNA sequences (GenBank accession number, NM_000821.3) using the NCBI nucleotide analysis tool Spidey. For each of the 96 DNA samples, PCR was performed with 10 ng of genomic DNA in a total reaction volume of 20 μl. All PCRs were performed by using GeneAmp PCR system 9700 (Applied Biosystems, USA) and used Ex Taq DNA polymerase (Takara Bio Inc, Japan).

In general, we performed PCR with an initial denaturation step of 94°C for 5 min, followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. These cycling reactions were followed by a final extension at 72°C for 5 min. For fragments 7, 9, and 10, we performed amplification as above, but increased the extension time of the 37 cycles from 1 to 2 min. We carried out SNP discovery by direct sequencing of the PCR products by using 96-capillary 3730*xl* DNA Analyzer (Applied Biosystems, USA). We sequenced all amplified fragments by two pairs of sequencing primers, except for fragments 5A and 5B, which were each amplified by only a pair of primers. All SNPs were detected by the Polyphred computer program (Nickerson et al. 1997) and were confirmed by sequencing both strands of each PCR product.

Statistical analyses

We calculated and tested genotype frequencies for each marker with the standard *Chi*-square test of Hardy–Weinberg Equilibrium (HWE) (Weir 1996). We used only polymorphisms with minor allele frequencies of greater than 0.1 for subsequent haplotype analyses. We estimated haplotypes and corresponding haplotype frequencies using the Expectation–Maximization (EM) algorithm (Slatkin and Excoffier 1996; Kitamura et al. 2002).

Association study

We performed both the Kruskal–Wallis and Mann–Whitney *U* tests to evaluate association between each of the polymorphisms with the warfarin maintenance-dose requirement of the 96 individuals. For the CAA

microsatellite in intron 6 of *GGCX*, we performed the Mann–Whitney *U* test to examine whether an absence or a presence of a particular allele or genotype of the CAA repeats is associated with the warfarin maintenance-dose requirement of our subjects.

We increased the number of individuals genotyped for a particular polymorphism if we detected possible association between that polymorphism with the warfarin maintenance-dose of the 96 individuals. We also genotyped more individuals for a cSNPs (rs699664) and a microsatellite (rs10654848) because previous studies (Shikata et al. 2004; Kimura et al. 2007) reported that these polymorphisms influence the warfarin dose requirement of Japanese individuals. In all cases, we PCR-amplified and direct sequenced the genomic region containing the particular polymorphism under investigation.

Results and discussion

Direct sequencing of an 18-kb genomic region corresponding to *GGCX*, using DNAs from 96 Japanese patients treated with warfarin, identified 49 genetic variations including 41 SNPs, seven insertion/deletion polymorphisms, and one microsatellite polymorphism. Six polymorphisms are found in the 5′ flanking region, seven in exons, 27 in introns, and nine in the 3′ flanking region of *GGCX*. On average, genetic variations were found in every 367 nucleotides. Figure 1 illustrates genomic organization of the 18-kb region and locations of all genetic variations, and their detailed information is summarized in Table 1.

Among the 49 polymorphisms we discovered in this genomic region, 32 variations—27 SNPs and five insertion/deletions—were not deposited in the public database, and are considered to be novel. In addition, 19 polymorphisms deposited in the dbSNP database were not polymorphic in the 96 Japanese individuals we used in this study. Genotype distributions, minor allele frequencies (MAF), and *P* value for the *Chi*-square test of HWE for all loci, except for *GGCX*-49, are shown in Table 2.

All insertion/deletion polymorphisms detected in this study except for *GGCX*-42 are biallelic (a presence or an absence of the particular inserted/deleted nucleotide(s)) but *GGCX*-42 was found to be a tri-allelic polymorphism of eight, nine, and ten repeats of nucleotide G. For *GGCX*-49, a microsatellite polymorphism at position -179 in intron 6, seven alleles and ten genotypes (Table 3) have been identified.

Among the seven polymorphisms located in the exonic regions, three cause amino-acid substitutions (Pro113Leu in exon 1, Arg325Gln in exon 8, and Val460Ile in exon 10). *GGCX*-46, an insertion of TAAA, is located in the untranslated region of exon 15, at one of the two concensus polyA+ addition signals of *GGCX*. However, whether this insertion alters the function of the poly A+ addition signal of *GGCX* remained unknown.

Results of *the Chi*-square test of HWE revealed that 39 of the 41 investigated SNPs, as well as all insertion/deletions, fit the law of HWE. Two SNPs that did not follow the Hardy–Weinberg equilibrium ($P \leq 0.05$), *GGCX*-1 and *GGCX*-33, are non-exonic SNPs. As summarized in Table 2, 18 polymorphisms have minor allele frequencies of greater than 0.1. With reference to the genotyping data, we eliminated six SNPs that are in absolute linkage disequilibrium (LD) with others, and used genotype information of the remaining 11 SNPs (including *GGCX*-42) and *GGCX*-49 (the microsatellite) for subsequent haplotype estimation by the EM algorithm. Twenty-one possible haplotypes of the 12 polymorphisms have been estimated, and ten of them that have frequencies of greater than 0.01 are listed in Table 4. We found that four major haplotypes (Haplotype 1–4) could cover 79% of the haplotypes in this genomic region.

For the association with the warfarin dosage, we screened primary results for the 96 Japanese subjects treated with warfarin by the Kruskal–Wallis and Mann–Whitney *U* tests (as summarized in Tables 2 and 3) and

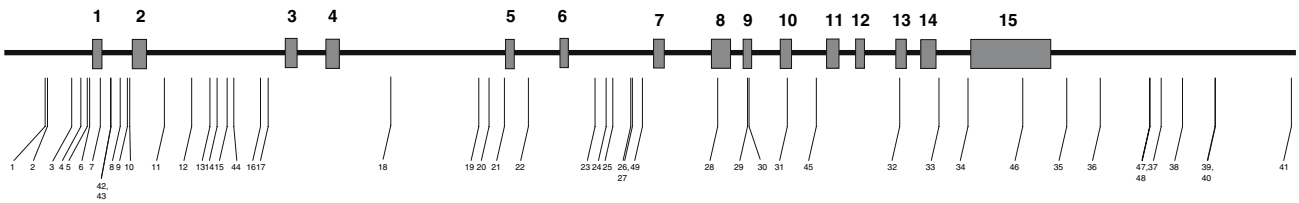

Fig. 1 Genomic organization of the human Gamma-glutamyl carboxylase gene (*GGCX*) and locations of the 49 polymorphisms detected in the 18-kb genomic region containing *GGCX* that we examined in this study. *Tall*, *grey boxes* illustrate exons, while *short*, *black boxes* represent introns. The numerical number on *top* of each *grey box* indicates the number of each exon

Table 1 Characterization of 49 genetic variants in the 18-kb genomic region containing *GGCX*. Both 5′ and 3′ sequences flanking the genetic variants are written in lower case letters, while the variants themselves were highlighted in capital letters

Position on contig	Polymorphism_ID	Location	Position*	5′ flanking sequence	Variation	3′ flanking sequence	dbSNP	Amino acid changes
64605065	*GGCX*-1	5′ flanking region	–556	tatcgatcctcccgcattgg	C/A	cttccaaactgttgggatta		
64605031	*GGCX*-2	5′ flanking region	–522	gggattacaagcgagagcag	T/G	gcccggcccctactgcttt		
64604791	*GGCX*-3	5′ flanking region	–282	aaggaagttggggaactcag	A/C	ctcttttcagtgtttaaaaa		
64604668	*GGCX*-4	5′ flanking region	–159	gctccaaggctccttgttcc	G/T	ccccctgccgccaagcaag		
64604551	*GGCX*-5	5′ flanking region	–42	gtcgggaggcggagcctagg	G/A	aagcaaattctcct[G/T]gcgg		
64604536	*GGCX*-6	5′ flanking region	–27	ctagg[G/A]aagcaaattctcct	G/T	gcggcctccgttcagacgcg		
64604447	*GGCX*-7	Exon 1	63	cgggtccgcgcggacctcgc	C/T	cagctcaggtaggcttagga		Pro12Leu
64604210	*GGCX*-8	Intron 1	–162	acattaggggatcgtggaga	G/T	ggggctgcactgcagggatg	rs10172544	
64604115	*GGCX*-9	Intron 1	–67	aagctctgagctgttggtgc	A/T	gtgatttctttgatttgagg	rs7568458	
64604080	*GGCX*-10	Intron 1	–32	ttgaggtggagcccaccccc	G/A	cacacatattttctccattt	rs1254898	
64603590	*GGCX*-11	Intron 2	288	cactgtaacctccgccaccc	G/A	ggttaaagcgattctcctcc		
64603281	*GGCX*-12	Intron 2	597	ccagaagactcagagaaaca	G/A	agcaggggccacagggacc	rs12714145	
64603063	*GGCX*-13	Intron 2	815	gtcacaacttgggggaggaa	G/A	aataggtggctgctggcatc		
64602989	*GGCX*-14	Intron 2	–851	tgtagtgcattggataatcc	G/A	ctcaataaataattaaccag		
64602877	*GGCX*-15	Intron 2	–739	ctcttctgtttattcccagg	G/A	tgttgacgaaatgcaactg		
64602496	*GGCX*-16	Intron 2	–358	ggcgtgagccaccgtgcctg	G/C	cctcccagagcactttctga	rs11254897	
64602416	*GGCX*-17	Intron 2	–278	acagtttttatagcattttg	T/G	gcatttctgtaatttaatcc		
64600790	*GGCX*-18	Intron 4	713	ttaaatagagatggggtctc	G/A	ctttgttgcccaggctggtc		
64599705	*GGCX*-19	Intron 4	–382	actctgctgttgagggtttt	C/G	attttttttaaagataata	rs17026452	
64599569	*GGCX*-20	Intron 4	–246	ctgatttgatccattagaac	A/C	aactcaactgggagctaaca		
64599327	*GGCX*-21	Intron 4	–4	caatgtttacctccctcggt	G/A	taggtctgtggacggtctgc		
64599068	*GGCX*-22	Intron 5	177	aatcccttaatctctgaatt	A/C	tcttatttgtcaaggaaaga	rs6738645	
64598162	*GGCX*-23	Intron 6	385	gaagatgggcactcactgtt	C/A	aggctctgacaaaaccatta		
64598045	*GGCX*-24	Intron 6	502	tattctctttcttcttccct	G/A	ttattttccagttcctgaag		
64597963	*GGCX*-25	Intron 6	584	agtgtcctctcactcatgac	C/T	tccgtggggaacgtcccttc	rs 762684	
64597708	*GGCX*-26	Intron 6	–339	gacactgtctctacaaaaaa	A/T	ttttt[T>A]aaaaaaacagctag	rs10196357	
64597702	*GGCX*-27	Intron 6	–333	gtctctacaaaaaa[A/T]ttttt	T/A	aaaaaaacagctaggcgtgg		
64596476	*GGCX*-28	Exon 8	85	gctggtgtcctactgccccc	G/A	aaggttgcaacaactgttgc	rs699664	Arg325Gln
64596071	*GGCX*-29	Exon 9	63	gacatgatggtgcactcccg	C/T	tcccaccagcacgtgaagat	rs2592551	Synonymous
64596047	*GGCX*-30	Exon 9	87	caccagcacgtgaagatcac	C/T	taccgtgatggccgcactgg	rs10179904	Synonymous
67595540	*GGCX*-31	Exon 10	91	gcctgcttcccaagtataat	G/A	tcactgagccccagatctac		Val460Ile
64594088	*GGCX*-32	Exon 13	48	tatacgacatcacctagccc	T/A	tcttgctacatgtacgtcta		Synonymous
64593581	*GGCX*-33	Intron 14	37	atttgtcattgccatcatat	G/A	ttggcaagcttggtaacttt		
64593210	*GGCX*-34	Intron 14	–21	aacaggagtttgaacttggt	C/T	ggctttttcctgtttttcag	rs2028898	

Table 1 continued

Position on contig	Polymorphism_ID	Location	Position*	5′ flanking sequence	Variation	3′ flanking sequence	dbSNP	Amino acid changes
64591948	*GGCX*-35	3′ flanking region	195	tctaaaaataagcttagta	T/A	ttggtattattctagccttt	rs13406935	
64591393	*GGCX*-36	3′ flanking region	750	ttattctttattattgaatg	G/A	cagctctgtaccttagccaa		
64591240	*GGCX*-37	3′ flanking region	1527	aacaaaagtcccaagagtta	C/T	ttgggagaagacacttgaaa		
64590974	*GGCX*-38	3′ flanking region	1793	agtatacatcctcctcattc	T/C	accctcaggatttggatctt		
64590575	*GGCX*-39	3′ flanking region	2192	catgctcattcccctaatgt	A/G	[G/A]ccaatatggttatctgcaa		
64590574	*GGCX*-40	3′ flanking region	2193	atgctcattcccctaatgt[A/G]	G/A	ccaatatggttatctgcaat		
64590334	*GGCX*-41	3′ flanking region	2433	caatggcgccatctcggctc	A/G	ctgcaacctctgcctcctgg		
64604343	*GGCX*-42	Intron 1	106	cccggtgaggcgggggggg	G/del	[T/-]cctctgtggggaaggggc		
64604342	*GGCX*-43	Intron 1	107	ccggtgaggcgggggggg[G/-]	T/del	cctctgtggggaaggggcg	rs11350741	
64602806	*GGCX*-44	Intron 2	–668	tgagaagcactcccagagca	CTTT/del	ctttctttctttctttctttt		
64595147	*GGCX*-45	Intron 10	–103	gctgacacaggccatttctt	CTT/del	ctattccctgtccttaggaa		
64592437	*GGCX*-46	Exon 15 (Untranslated)	752	agacttcgtctcaaaataaa	ins/TAAA	taaaagacttcgtctcaaaa	rs10691423	3′ untranslated region
64590770	*GGCX*-47	3′ flanking region	1373	tggctgtggcacagggtccc	C/del	[T/-]gattactttgtaagaatgg		
64590769	*GGCX*-48	3′ flanking region	1374	ggctgtggcacagggtccc[C/-]	T/del	gattactttgtaagaatggg		
64597526	*GGCX*-49	Intron 6	–179	gacagagtgaaacttcgttt	(CAA)n	cctagaggagtgtcttcatg	rs10654848	

* If the polymorphism is located in exon, the numerical value indicates how many nucleotides away from the first base of the exon the particular polymorphism is located . However, if the polymorphism is located in intron, the numerical value indicates the distance of that particular polymorphism away from the nearest exon. A positive number indicates the number of nucleotides away from the previous exon, while a negative number indicates the number of nucleotides away from the next exon

Table 2 Genotype distributions and minor allele frequencies (MAF) of the 48 polymorphisms detected. Also shown are the *P* value of the *Chi*-square test of Hardy–Weinberg Equilibrium (HWE) of genotypes at each locus, as well as *P* values of the Kruskal–Wallis and Mann–Whitney *U* tests of the association between each polymorphism and the warfarin maintenance-dose requirements of the 96 Japanese individuals

Polymorphism_ID	Genotype distribution			MAF	HWE Chi^2 test *P* value	Kruskal–Wallis test *P* value	Mann–Whitney *U* test *P* values	
	11	12	22				11 vs 12 + 22	22 vs 11 + 12
GGCX-1*	76	9	2	0.075	0.019	0.638	0.979	0.380
GGCX-2	81	8	0	0.045	0.657	0.524	0.524	NA
GGCX-3	93	1	0	0.005	0.959	1.000	1.000	NA
GGCX-4	88	5	0	0.027	0.790	0.666	0.666	NA
GGCX-5	88	1	0	0.006	0.957	0.968	0.968	NA
GGCX-6	86	1	0	0.006	0.957	0.276	0.276	NA
GGCX-7	86	1	0	0.006	0.957	0.276	0.276	NA
GGCX-8	38	41	15	0.378	0.484	0.788	0.842	0.585
GGCX-9	37	42	15	0.383	0.597	0.788	0.838	0.585
GGCX-10	69	27	0	0.141	0.109	0.455	0.455	NA
GGCX-11	83	2	0	0.012	0.913	0.377	0.377	NA
GGCX-12	38	41	16	0.384	0.391	0.857	0.804	0.711
GGCX-14	95	1	0	0.005	0.959	0.254	0.254	NA
GGCX-15	95	1	0	0.005	0.959	1.000	1.000	NA
GGCX-16	69	27	0	0.141	0.109	0.455	0.455	NA
GGCX-17**	92	4	0	0.021	0.835	0.022	0.022	NA
GGCX-18	95	1	0	0.005	0.959	0.254	0.254	NA
GGCX-19	95	1	0	0.005	0.959	1.000	1.000	NA
GGCX-20	94	2	0	0.010	0.918	0.842	0.842	NA
GGCX-21	94	2	0	0.010	0.918	0.217	0.217	NA
GGCX-22	37	44	15	0.385	0.750	0.691	0.729	0.534
GGCX-23	86	10	0	0.052	0.590	0.190	0.190	NA
GGCX-24	86	9	0	0.047	0.628	0.163	0.163	NA
GGCX-25	48	39	9	0.297	0.792	0.883	0.619	0.896
GGCX-26	83	12	1	0.073	0.460	0.419	0.313	0.279
GGCX-27	94	2	0	0.010	0.918	0.770	0.770	NA
GGCX-28	45	41	8	0.303	0.754	0.767	0.583	0.536
GGCX-29	45	42	9	0.313	0.859	0.763	0.466	0.896
GGCX-30	81	12	0	0.065	0.506	0.429	0.429	NA
GGCX-31	94	1	0	0.005	0.959	0.250	0.250	NA
GGCX-32	94	1	0	0.005	0.959	0.985	0.985	NA
GGCX-33*	92	2	1	0.021	0.000	0.273	0.149	0.157
GGCX-34	45	41	6	0.288	0.406	0.503	0.395	0.317
GGCX-35	79	13	0	0.071	0.466	0.221	0.221	NA
GGCX-36	91	1	0	0.005	0.958	0.984	0.984	NA
GGCX-37	38	41	14	0.371	0.593	0.493	0.820	0.311
GGCX-38	93	1	0	0.005	0.959	0.970	0.970	NA
GGCX-39	94	2	0	0.010	0.918	1.000	1.000	NA
GGCX-40	83	12	0	0.063	0.511	0.403	0.403	NA
GGCX-41	95	1	0	0.005	0.959	0.254	0.254	NA
GGCX-42***	38	43	15	0.078	0.627	0.689	0.730	0.534
GGCX-43	38	43	15	0.380	0.627	0.689	0.730	0.534
GGCX-44	44	41	11	0.328	0.759	0.506	0.336	0.761
GGCX-45	48	40	8	0.292	0.934	0.821	0.531	0.891
GGCX-46	37	44	15	0.385	0.750	0.691	0.729	0.534

Table 2 continued

Polymorphism_ID	Genotype distribution			MAF	HWE Chi^2 test P value	Kruskal–Wallis test P value	Mann–Whitney U test P values	
	11	12	22				11 vs 12 + 22	22 vs 11 + 12
GGCX-47	46	41	9	0.307	0.975	0.763	0.466	0.896
GGCX-48	44	38	8	0.300	0.960	0.798	0.694	0.522

* Polymorphisms in Hardy–Weinberg disequilibrium

** SNP showing significant association with the warfarin maintenance dose-requirements in the 96 Japanese individuals

*** For *GGCX*-42, three alleles (8, 9, and 10 repeats of G) and three genotypes (8G/9G, 9G/9G, and 9G/10G) were observed; genotypes 11, 12, and 22 in the above table indicate genotypes 8G/9G, 9G/9G, and 9G/10G respectively

Table 3 Genotype distributions of *GGCX*-49 (Microsatellite Intron 6 -179 (CAA)n) and association between different alleles and genotypes of the microsatellite with warfarin maintenance-dose requirement of the 96 Japanese individuals

	Alleles/genotypes	96 individuals	
		Frequencies	Mann–Whitney U test P values
Alleles or number of (CAA) repeats	8	0.005	1.000
	10	0.594	0.756
	11	0.359	0.794
	12	0.005	0.254
	13	0.016	0.711
	14	0.016	0.703
	15	0.005	0.279
Genotypes	8 > 10	0.010	1.000
	10 > 11	0.396	0.783
	10 > 13	0.021	0.652
	10 > 14	0.010	0.104
	11 > 11	0.146	1.000
	11 > 13	0.010	1.000
	11 > 14	0.010	0.254
	11 > 15	0.010	0.279
	12 > 14	0.010	0.254

* Each test was performed by grouping patients into two groups every time, with or without the tested allele/genotype

found one, *GGCX*-17, of the 49 polymorphisms revealed a possible association with a P value of 0.022 with the warfarin maintenance dose. In order to further evaluate the significance of this association, we genotyped an additional 732 Japanese individuals for this SNP, but no association was detected ($P = 0.394$).

In addition, we further investigated rs699664 G>A, because it causes a substitution of Gln for Arg at codon 325 and was reported previously by Kimura et al. (2007) to significantly relate to the warfarin maintenance dose of their 93 Japanese patients ($P = 0.022$). However, we genotyped 362 Japanese individuals and found no association ($P = 0.636$) between this cSNP and the warfarin maintenance dose. Similarly, genotyping results of 365 individuals at the CAA microsatellite of *GGCX* revealed no association with the warfarin maintenance dose of our patients.

GGCX, which plays an important role in the vitamin K cycle, is a candidate gene for the investigation of inter-individual differences in the warfarin maintenance dose requirement. Although warfarin has been the most frequently prescribed oral anticoagulant in prevention of thromboembolism, administration of warfarin to achieve sufficient suppression of thrombosis without causing undesired bleeding has remained a challenge for physicians, owing to a large variation in the inter-individual dose requirement (Daly and King 2003). The findings that recently known genetic and environmental factors only

Table 4 Haplotypes and corresponding frequencies as estimated by EM-algorithm (Kitamura et al. 2002) by using information of 12 polymorphisms. There are ten haplotypes with haplotype frequencies of greater than 0.01, as shown in this table

Haplotype_ID	1	2	3	4	5	6	7	8	9	10	11	12	Haplotype frequency
	GGCX-42	*GGCX-43*	*GGCX-10*	*GGCX-13*	*GGCX-16*	*GGCX-22*	*GGCX-25*	*GGCX-49*	*GGCX-29*	*GGCX-45*	*GGCX-46*	*GGCX-47*	
1	9	T	G	G	G	A	C	10	C	CTT	–	C	0.260
2	9	–	G	A	G	C	T	11	T	–	TAAA	–	0.203
3	8	T	G	G	G	A	C	10	C	CTT	–	C	0.188
4	9	T	A	G	C	A	C	10	C	CTT	–	C	0.141
5	10	–	G	A	G	C	T	11	T	–	TAAA	–	0.068
6	9	–	G	G	G	C	C	11	C	CTT	TAAA	C	0.047
7	9	T	G	G	G	A	C	11	C	CTT	–	C	0.021
8	9	–	G	A	G	C	C	11	T	–	TAAA	–	0.016
9	9	–	G	G	G	C	C	14	C	CTT	TAAA	C	0.016
10	9	–	G	A	G	C	T	13	T	CTT	TAAA	–	0.016

* Symbol "–" means deletion or absence of the particular allele

partially explain inter-individual variability in warfarin maintenance-dose requirements (Loebstein et al. 2005; Schwarz and Stein 2006) indicate the need to uncover other unknown genetic determinants of the response to warfarin for better administration of this oral anticoagulant.

Although we failed to identify any polymorphisms that are the genetic determinants of the warfarin maintenance dose of our Japanese subjects in the current study, the high-resolution SNP map within the 18-kb genomic region containing *GGCX* should serve as a useful resource for further pharmacogenetic studies for various drugs in whose pharmacological pathway GGCX may be involved.

Acknowledgments We thank all members of the Laboratory for Pharmacogenetics, SRC, RIKEN, Japan, for their helpful contribution to the completion of this study. We also thank Drs Yozo Ohnishi, Aritoshi Iida, Michiaki Kubo, and Susumu Saito for stimulating discussion and helpful comments.

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