

Table 3. Frequencies of haplotypes constructed from six SNPs

SNP1 TG	SNP2 AG	SNP3 GA	SNP4 TC	SNP5 CT	SNP6 GA	Frequency
G	G	A	C	C	G	0.155
T	G	G	T	T	G	0.114
T	G	A	C	C	G	0.091
T	G	A	C	C	A	0.074
T	G	G	C	C	A	0.060
T	A	A	C	C	G	0.059
G	A	A	T	T	G	0.053
T	A	G	C	C	A	0.053
G	A	A	C	C	A	0.049
G	G	A	C	C	A	0.046
T	A	A	C	C	A	0.038
T	A	A	T	T	G	0.037
T	G	A	T	T	G	0.032
G	A	A	C	C	G	0.026
T	A	G	T	T	G	0.021
T	G	G	C	T	G	0.019
G	A	G	T	T	G	0.016
T	G	G	C	C	G	0.013
G	A	G	C	C	G	0.012
T	A	G	C	C	G	0.012
G	A	A	T	C	G	0.009
T	A	A	T	C	G	0.009
Total						1.000

human *CL-PI* locus and human diseases, especially in association studies between specific polymorphisms and susceptibility to innate immunity disease or atherosclerosis.

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ONLINE MUTATION REPORT

Common origin of the Val30Met mutation responsible for the amyloidogenic transthyretin type of familial amyloidotic polyneuropathy

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The amyloidogenic transthyretin (TTR) type of familial amyloidotic polyneuropathy (FAP [MIM 176300], <http://www.ncbi.nlm.nih.gov/Omim/>) is the most common form of hereditary systemic amyloidosis.¹⁻³ It is well known that amyloidogenic TTR resulting from gene mutations is a major constituent of amyloid deposits in tissues of patients with FAP.⁴ To date, more than 80 mutations of the TTR gene have been associated with human amyloidosis.⁵ Of those mutations, a mutation changing valine at amino acid 30 to methionine (Val30Met) is the most common,¹⁻³ and only patients with this mutation are found in large foci throughout the world.^{1, 2}

The clinical manifestations of the TTR Val30Met type of FAP in Japan and Portugal are as follows: (1) the disorder is inherited as an autosomal dominant trait with equal sex distribution and high penetrance; (2) the age at onset is late twenties to early forties; (3) polyneuropathy with sensory dissociation starts in the legs and progresses in an ascending fashion; (4) manifestations of various autonomic dysfunctions such as severe orthostatic hypotension, disturbed bowel movement with constipation and diarrhoea, impotence, and urinary incontinence invariably appear during the course of the disease; and (5) amyloid deposition in loco causes dysfunction of various organs.^{1-3, 6} In contrast, the clinical profile of Swedish patients is different: the average age at onset is the middle fifties, and penetrance of the disease is very low. Also, the disease progresses more slowly in Swedish patients than in Japanese and Portuguese patients.⁷

The disorder was first described in Portugal in 1952.⁸ In the 1960s, large foci of patients were found in Japan and Sweden.^{9, 10} In Japan, two large foci of TTR Val30Met type FAP exist: one in Kumamoto prefecture of Kyushu, and the other in Nagano prefecture on the Mainland of Japan.¹ However, these two foci are geographically distant, and a consanguineous relationship between the foci has not been identified. The issue of whether there is a common origin for a mutant allele in the two foci has not been resolved. Furthermore, Continho¹¹ hypothesised that a mutant allele in the Portuguese kindred could be the origin of the mutation for FAP foci throughout the world, including Japan, Europe, North and South America, and Africa. This hypothesis was based only on well-known historical bonds, and thus far it has not been scientifically tested.

Recently, estimation of haplotype in the absence of DNA of related individuals became possible by means of the maximum-likelihood method algorithm.¹² In addition, the human genome project has continuously provided human genome resources. Almost 200,000 reliable single-nucleotide polymorphisms (SNPs) for the entire genome of the Japanese population are now available (IMS JSNP database, <http://snp.ims.u-tokyo.ac.jp/index.html>). Microsatellite information is now also available (GenBank, <http://www.ncbi.nlm.nih.gov/Entrez/>); assay of this information provides a powerful method for determining the origin of the gene because heterozygosity is usually much higher than that for SNPs. Statistical software can be used to construct a haplotype by using SNPs and microsatellite information together (Arlequin, <http://lgb.unige.ch/arlequin/>). Thus, we decided to reanalyse the origin of Val30Met mutation with the newly developed methods and resources.

Key points

- Our work aimed to identify the origin of Val30Met mutation, responsible for the amyloidogenic transthyretin (TTR) type of familial amyloidotic polyneuropathy (FAP) found in Japan and Europe as foci.
- We analysed the TTR gene haplotype of FAP patients from Japan, Portugal, Spain, and Sweden, and of Japanese and Caucasian control subjects with four single nucleotide polymorphisms (SNPs) and five microsatellite markers in the neighbouring gene region, spanning 215 kb (loci 1-9).
- We observed that the disease-causing haplotype is the same in two major foci in Japan, indicating the existence of a common founder.
- By comparing haplotype among foci, we obtained results indicating that there could be a common founder for Japanese and Portuguese patients, and for Portuguese and Spanish patients, but not for Swedish or other patients.
- These data, plus the history of 16th century trade, lead to the plausible hypothesis of a mutant allele of a Portuguese kindred being the origin of the mutation in most Japanese FAP patients.

We first collected DNA samples from five foci—two foci in Japan and one each in Portugal, Majorca Island (Spain), and Sweden—and then analysed haplotype structures with four SNPs and five microsatellite markers covering a 215 kb TTR gene region. We observed major haplotypes close to 50% in frequency in each focus of the world, which indicated that each focus originated from one founder because the disease is inherited in an autosomal dominant fashion. Furthermore, we obtained support for the hypothesis that the origin of the mutation is common in the Spanish, Portuguese, and Japanese foci but not in the Swedish foci.

Abbreviations: FAP, familial amyloidotic polyneuropathy; SNPs, single nucleotide polymorphisms; TTR, transthyretin

METHODS

Study subjects

A total of 100 DNA samples were collected from FAP patients with *TTR* Val30Met in four countries. In Japan, in addition to patients in the two major foci (16 patients from Kumamoto and two from Nagano), 20 patients from other locations throughout the country agreed to participate in this study. In Europe, 21 samples from Majorca Island (Spain), 18 samples from Portugal, and 23 samples from Sweden were collected. A total of 54 healthy volunteers in Asahikiawa Medical College were recruited to provide DNA samples for haplotype analysis of the Japanese population, and 96 Caucasian DNA samples were purchased from the Coriell Cell Repository (Coriell Institute, Camden, NJ, USA). The Ethical Committee of Kumamoto University School of Medicine approved this study, and all patients and Japanese volunteers gave written informed consent.

Microsatellite genotyping

We selected five informative microsatellite sequences (L1, L2, L4, L8, and L9) near the *TTR* gene region from the National Center for Biotechnology Information (NCBI) database GenBank (fig 1). PCR primers were designed to flank the repeat sequences for the analysis. The PCR primers were as follows: L1 forward: 5'-TGCAACGAAGCTTCAAGAGA-3'; L1 reverse: 5'-AAGCAAATTGCAATGGGAAG-3'; L2 forward: 5'-AGGCAGTGGGATAGTCATGG-3'; L2 reverse: 5'-GGATTCCCTGGGCTCATTAT-3; L4 forward: 5'-TTCCTCTGGCCGACTTATT-3'; L4 reverse: 5'-TAGTGCTCCAAACGGACTT-3'; L8 forward: 5'-AACCTGGGAGATAGAGCTTGC-3'; L8 reverse: 5'-TCTCCTTAATCTAAGAAAGCCACAT-3'; L9 forward: 5'-GGCCGTACAGGTCATAGAA-3'; L9 reverse: 5'-CCACCAGAGGAAACCAACCT-3'. Fluorescence-labeled primers were used for genotyping. PCR was performed in a volume of 7.5 μ l containing 20 ng of genomic DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μ M dNTPs, each primer at 5 pmol, and 0.25 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). PCR amplification was performed for 40 cycles at 95°C for 30 s, at 53–60°C for 30 s, and at 72°C for 30 s, depending on the region analysed, with a final extension step of 5 min at 72°C in a Gene Amp PCR9700 System (Applied Biosystems, Foster City, CA). Electrophoresis was performed with an ABI 310 DNA sequencer; the data were extracted by GeneScan Analysis software (Applied Biosystems, Foster City, CA).

SNP genotyping

Four SNPs—L3, L5, L6, and L7—were identified from the IMS-JST JSNP database (fig 1). PCR was performed in a volume of 12.5 μ l containing 20 ng of genomic DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μ M dNTPs, each primer at 10 pmol, and 0.25 U AmpliTaq Gold polymerase. PCR amplification was performed for 40 cycles at 94°C for 30 s, at 49–62°C for 30 s, and at 72°C for 30 s, depending on the region analysed, with

a final extension step of 5 min at 72°C in a Gene Amp PCR9700 System (Applied Biosystems, Foster City, CA). The sequencing reaction and electrophoresis were performed with the BigDye Terminator kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol.

Haplotype estimation and statistical analysis

Haplotype analysis was done by using the Arlequin program, which is software for population genetic data analysis, and SNPalyze (DYNACOM, Mobara, Chiba, Japan). Maximum-likelihood haplotype frequencies were computed using an expectation-maximisation (EM) algorithm. This procedure is an interactive one aimed at obtaining maximum-likelihood estimates of haplotype frequencies from multilocus genotype data when the gametic phase is unknown. In this case, simple gene counting is not possible because several genotypes are possible for individuals who are heterozygous at more than one locus. The computation of the EM algorithm involves the following: start with arbitrary estimates of haplotype frequencies; use these estimates to compute expected genotype frequencies for each phenotype, if one assumes a Hardy-Weinberg equilibrium (the E-step); use the relative genotype frequencies as weights for their two constituent haplotypes in a gene-counting procedure, which leads to new estimates of haplotype frequencies (the M-step); repeat steps E and M until the haplotype frequencies reach equilibrium.¹²

RESULTS AND DISCUSSION

Nine polymorphisms were employed in the present study. Of those, five (L1, L2, L4, L8, and L9) are microsatellite markers (fig 1). L2 and L4 were in the 5'-flanking region of the gene, L1 was located about 90 kb upstream of L2, and L8 was about 103 kb downstream of L7. Thus, the gene region analysed covered 215 kb (fig 1). The heterozygosity of microsatellite markers of Japanese and Caucasian controls is shown in table 1. All but L2, whose name is D18S36, are anonymous in the GenBank database. All markers except L1 showed similar heterozygosities in Japanese and Caucasian subjects. Four SNPs used in this study (L3, L5, L6, and L7) were obtained from the Japanese SNP database (IMS-JST JSNP). Two of those (loci 3 and 5) were located in the 5'-flanking region, and the remaining two (loci 6 and 7) were located in intron 3, as shown in fig 1 and table 2. Almost no ethnic difference in allele frequency between Japanese and Caucasian control subjects was found. The Val30Met mutation occurs in exon 2 (fig 1).

All nine polymorphisms were employed to construct haplotypes for Japanese patients and controls. To clarify the origin of the mutant allele, Japanese patients were classified into three groups: 16 patients from Kumamoto, two from Nagano, and 20 from the rest of Japan. As shown in table 3, patients from Kumamoto showed a major haplotype frequency of 40% (which is close to 50%), but the same haplotype in the controls was very rare, in fact, it was

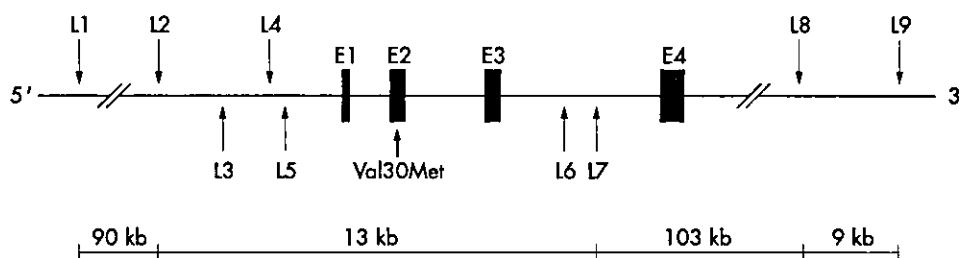


Figure 1 Genomic structure and locations of polymorphic sites used in the human *TTR* gene. Boxes indicate exons; horizontal lines represent introns and the 5' upstream and 3' downstream regions of the gene. Downward arrows indicate locations of microsatellite markers, and upward arrows indicate locations of SNPs. The thick upward arrow represents the position of the Val30Met mutation.

Table 1 Heterozygosity of microsatellite markers above Japanese and Caucasian subjects

	Japanese	Caucasian
L1	0.497	0.704
L2	0.766	0.702
L4	0.349	0.448
L8	0.737	0.761
L9	0.871	0.856

undetectable by our method. The most frequent haplotype (9.6%) in the controls was different in loci 1 and 9 from the major haplotype of the patients. When a haplotype exists with close to 50% frequency in patients of one focus of an autosomal dominant disease, the allele could be considered a disease-causing one, and the haplotype, a founder haplotype. Thus, the haplotype, 237-307-T-271-C-G-A-299-311, actually represents the disease-causing allele in this group. Although only two patients from Nagano were available for analysis, which is not sufficient for haplotype construction, it is evident from genotype data that they shared exactly the same haplotype with the Kumamoto patients (table 3). Furthermore, in the other Japanese patients the same haplotype had a frequency of 31.8%, with frequencies of other haplotypes being less than 5%.

To determine whether all major FAP foci throughout the world could be derived from a Portuguese mutant allele,¹¹ we analysed the haplotype pattern of patients from Portugal, Spain, and Sweden. We found that all European foci have their own major haplotypes, whose frequencies were close to 50% (40.9-47.6%), which indicated that these haplotypes represent a disease-causing allele, as for the Japanese focus (table 4). When the major haplotype of Portuguese patients was compared with that of Japanese patients, all loci except L8 were exactly the same, which indicated that at least the 103 kb region (loci 1-7) was common between the two populations. Also, comparison of Portuguese and Spanish patients revealed that at least the 116 kb region (loci 2-8) was common. However, Swedish patients shared only the 13 kb region (loci 2-7), which is also a major haplotype in the Caucasian controls, with other foci of patients.

Several major issues remain unsettled in the field of research on FAP caused by the Val30Met mutation of the *TTR* gene. First, although large foci of patients are located in several restricted areas of the world, why is only the Val30Met mutation found in those foci, even though more than 80 different mutations of *TTR* gene have been identified?⁵ Second, does any common founder exist among the large foci of patients throughout the world? Third, what causes the significant differences in clinical symptoms among FAP patients with the Val30Met mutation compared with FAP patients with other mutations? For example, the age at onset of the disease in patients in Sweden is much later than that in other countries such as Japan and Portugal.^{3, 7, 13}

To answer the second question, haplotype analysis via gene polymorphisms has been performed to clarify the founder effect. Several researchers have discussed the origin of the *TTR* Val30Met gene in FAP patients from Japan,¹⁴ North America,¹⁵ and Europe.^{16, 17} All these data were analysed by using six or seven SNPs inside the gene, a region of about 7.0 kb, and the haplotype was determined by pedigree analysis. Yoshioka *et al*¹⁴ analysed eight families, including two from each major focus in Japan. They concluded that the mutation had a multiple origin because two isolated families, not from the two large foci, have different haplotypes, although the remaining six have the same haplotype, which was named "haplotype I". Li and Sommer¹⁵ analysed six unrelated FAP patients in North America and performed haplotype analysis in the absence of DNA samples for relatives. They found four different haplotypes and thus agreed with a multiple origin hypothesis. For Europe, however, Almeida *et al*¹⁶ and Reilly *et al*¹⁷ found only haplotype I in patients from Portugal, Spain, and Sweden. In the present study, we used, in addition to four SNPs (two the same as those analysed before by researchers), four highly polymorphic microsatellite markers covering 215 kb of the *TTR* gene region. A microsatellite repeat is generally considered to have a relatively high mutation rate when compared with SNPs, but its mutation rate is estimated to be about 10^{-3} per locus per generation,¹⁸ which is negligibly low. If the duration of one generation is assumed to be 30 years, a mutation at a certain microsatellite locus would occur at a rate of only one in 30,000 years. In view of the high heterozygosity of microsatellite markers, such a microsatellite would be a powerful marker for our study.

To determine the haplotype profile in the general population, 54 Japanese and 96 Caucasian control subjects underwent genotyping, and haplotypes were constructed. As expected, haplotypes constructed with all nine polymorphisms had a low frequency, the highest frequencies being 9.6% in Japanese and 9.5% in Caucasians, which indicated recombination in this 215 kb region, which occurred over a substantial time period, as is usual (table 4).

In diseases inherited in an autosomal dominant fashion, an allele responsible for the disease in the patient population would theoretically have a frequency of 50%. The fact that the frequency of the major haplotype observed in the Kumamoto focus (237-307-T-271-C-G-A-299-311) was 40%, which is considered to be close to 50%, whereas that of Japanese controls was extremely low, indicates that the haplotype represents the disease-causing allele. Although only two patient samples were available from Nagano, the location of another major focus of the disease in Japan, genotype data revealed that they had exactly the same haplotype as that of the Kumamoto patients (table 3). These data strongly support the existence of a relatively recent common founder. As for other FAP patients from the rest of Japan,¹⁹ a majority of these cases were thought to result from an independent mutational event;²⁰ however, an unexpectedly high frequency, 31.8%, of the same haplotype was found, which

Table 2 Positions and allele frequencies of SNPs

SNP	ID	Position	Allele frequency	
			Japanese	Caucasian
L3	IMS-JST 118368	-2015T>C	0.712	0.665
L5	IMS-JST 118365	-1131C>T	0.981	0.964
L6	IMS-JST 118364	IVS3+1242C>A	0.750	0.660
L7	IMS-JST 152576	IVS3+1753T>G	0.713	0.670

Table 3 Major haplotype frequencies for Japanese patients and controls

Population	L1	L2	L3	L4	L5	L6	L7	L8	L9	Frequency
Kumamoto patients	237	307	T	271	C	G	A	299	311	0.400
	235	307	T	271	C	G	A	299	327	0.050
	235	311	T	271	C	G	A	305	311	0.050
Nagano patients	237/237	305/307	T/T	271/271	C/C	G/G	A/A	299/301	311/315	
	237/237	305/307	T/T	271/271	C/C	G/G	A/A	299/301	311/315	
Patients from other locations	237	307	T	271	C	G	A	299	311	0.318
	237	307	T	271	C	G	A	291	333	0.045
	237	307	T	271	C	G	A	299	317	0.045
	237	307	T	271	C	G	C	299	311	0.045
	237	305	T	271	C	G	A	301	315	0.045
Japanese controls	241	307	T	271	C	G	A	299	325	0.096
	241	307	T	271	C	G	A	299	329	0.075
	241	307	C	269	C	G	C	299	311	0.038
	241	307	T	271	C	G	A	303	311	0.038
	241	305	T	271	C	G	A	299	327	0.029
	241	307	T	271	C	G	A	305	311	0.029
	241	309	T	271	C	G	A	307	311	0.019
	241	313	C	269	C	T	A	299	329	0.019
	241	307	T	271	C	G	C	299	317	0.011
	241	307	T	271	C	G	A	301	329	0.011

suggests that most were also derived from the same founder of the foci, rather than from independent sporadic mutations.

Each focus of European FAP patients has its own major haplotype, with a frequency close to 50% (40.9–47.6%) (table 4). The major haplotypes for Portuguese and Japanese patients are the same except for locus 8; in other words, at least the 103 kb region from locus 1 to locus 7 is common. One plausible explanation for this would be that the Portuguese Val30Met mutation was brought to Japan in recent times. Portuguese merchants arrived in Japan during the 16th century, and active trading was pursued in all areas in Kyushu (the island on which Kumamoto is located) until the beginning of the 17th century, when Japan became a closed country.¹¹ The most likely explanation is thus as follows: in the early phase of transfer of the allele into the Japanese population, recombination with the Japanese allele (L8–L9; 299–311, which was found in Japanese controls) occurred once between L7 and L8, after which the allele with the Val30Met mutation spread to Nagano and other areas of Japan.

Comparison of the major haplotypes for Portuguese and Spanish patients showed that a 116 kb region (loci 2–8) was common. This could also be explained by the existence of a common founder. Swedish patients, however, had only a 13 kb region (loci 3–7) in common with patients of other foci. Although the possibility of a common founder of Swedish and Portuguese cases cannot be ruled out, the fact that the

haplotype of this 13 kb region is the same as the major haplotype found in Caucasian controls might indicate that the Val30Met mutation of the Swedish focus is due to an independent mutational event. However, further molecular genetic studies have to be performed focusing on the Swedish cases because of a trade history including the Swedish Viking invasion of Portugal in the 10th century, and because far milder symptoms occur in Swedish patients than in Japanese or Portuguese patients. The cause of the discrepant clinical phenotypes of Swedish patients and patients from other foci—the third question mentioned above—could be a difference in genetic background such as the existence of modifying gene(s) and/or other known or unknown mechanisms. The answer to this question would be quite valuable for development of therapeutic measures for FAP patients.

Because the haplotypes of Spain and Portugal are somewhat diverse and that of Japan has a single disease-causing allele, the European haplotype could be older than the Japanese haplotype. This suggestion would support the hypothesis that the Portuguese mutant allele was brought to Japan, rather than transferred in an opposite direction, from Japan to Portugal.

In summary, our data provide the molecular evidence that the Val30Met mutation that is widespread among patients in Portugal, Spain, and Japan, results from an ancient mutation of common origin rather than from multiple recurrent mutational events in a common haplotype. The data also

Table 4 Major haplotype frequencies for foci of FAP patients and for controls

Population	L1	L2	L3	L4	L5	L6	L7	L8	L9	Frequency
Kumamoto patients	237	307	T	271	C	G	A	299	311	0.400
	235	307	T	271	C	G	A	299	327	0.050
	235	311	T	271	C	G	A	305	311	0.050
Japanese controls	241	307	T	271	C	G	A	299	325	0.096
	241	307	T	271	C	G	A	299	329	0.075
	241	307	C	269	C	G	C	299	311	0.038
	241	307	T	271	C	G	A	303	311	0.038
	241	305	T	271	C	G	A	299	327	0.029
Portugese patients	237	307	T	271	C	G	A	291	311	0.438
Spanish patients	235	307	T	271	C	G	A	291	321	0.476
Swedish patients	241	307	T	271	C	G	A	303	329	0.409
	237	307	T	271	C	G	A	291	311	0.087
Caucasian controls	241	307	T	271	C	G	A	299	329	0.095
	241	307	T	271	C	G	A	299	325	0.026
	241	307	T	271	C	G	A	299	331	0.026
	241	313	C	269	C	T	C	303	319	0.026

clearly demonstrate that microsatellite analysis must be applied in combination with SNPs when the origin and distribution of mutations are studied.

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A1166C variant of angiotensin II type 1 receptor gene is associated with severe hypertension in pregnancy independently of T235 variant of angiotensinogen gene

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Abstract Hypertension in pregnancy (HP) is a multifactorial disease manifested due to a complex combination of environmental factors and several predisposing genes including factors in the renin angiotensin system. The aim of this study was to assess the association between the A1166C variant of the angiotensin II type 1 receptor (AT1) gene and severe HP. We carried out association studies and multivariate analyses including other candidate causal factors of HP such as the M235T variant of the angiotensinogen (AGT) gene, prepregnancy body mass index (BMI), and family history of hypertension in Japanese subjects. One hundred and fourteen patients with severe HP and 291 normal pregnancy controls were genotyped. Among primiparous subjects, the frequency of “AC+CC genotype of AT1” was significantly higher in severe HP than in the controls. A multivariate analysis with “AC+CC genotype of AT1” and “TT genotype of AGT” revealed that these were independently associated with primiparous severe HP. However, when “family history of hypertension” and “pregnancy BMI ≥ 25 ” were added as factors examined in the multivariate analysis, only “TT

genotype of AGT” and “family history of hypertension” were found to be independent potent factors. The present results suggest that the C1166 allele of the AT1 gene may be concerned with the predisposition to essential hypertension independently of the T235 allele of the AGT gene.

Keywords Preeclampsia · Polymorphism · Multivariate analysis · Body mass index · Family history

Introduction

Hypertension in pregnancy (HP), including preeclampsia (PE), a proteinuric and/or edematous type of HP, is a major contributor to maternal, fetal, and neonatal morbidity and mortality. HP occurs in about 5% of pregnancies and is considered to be a multifactorial disease manifested due to a complex combination of environmental factors and several predisposing genes whose products likely interact with each other.

Genes coding for components of the renin angiotensin system involved in blood pressure regulation and vascular smooth muscle cell proliferation are considered to be candidate genes for risk factors for HP as well as essential hypertension. A molecular variant of the angiotensinogen (AGT) gene encoding threonine (T235) instead of methionine (M235) at residue 235 in exon 2 (Jeunemaitre et al. 1992) was shown to be associated with essential hypertension, and this association has been confirmed by some other investigators (Hata et al. 1994; Caulfield et al. 1994). An association with this allele was also demonstrated for primigravid PE in Caucasians (Ward et al. 1993) and HP in Japanese (Kobashi et al. 1999). A promoter mutation of G(-6)A is present in 99% of women with the M235T mutation, and *in vitro* studies have suggested that the A(-6) may cause elevated AGT expression *in vivo* (Inoue et al. 1997). Recently, it was reported that T235 might be

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concerned with abnormal pregnancy-induced spiral artery remodeling occurring early in pregnancy, which might lead to reduced uteroplacental blood flow and initiate the cascade of events leading to PE (Morgan et al. 1999).

On the other hand, in 1994, a polymorphism of the angiotensin II type 1 receptor (AT1) gene, namely an adenine/cytosine (A/C) base substitution at position 1166 located in the 3' untranslated region, was identified and an increased prevalence of the C allele in hypertensive disorders was found (Bonnardeaux et al. 1994). This association has been confirmed by some investigators (Szombathy et al. 1998; Jiang et al. 2001) but not by others (Schmidt et al. 1997; Takami et al. 1998). More recent studies further demonstrated associations between the C1166 allele of the AT1 gene and HP in Caucasian (Nalogowska-Glosnicka et al. 2000) and Polish (Seremak-Mrozikiewicz et al. 2000) subjects. However, the C1166 allele of the AT1 gene has not yet been confirmed to be an independent variant when other possible risk factors are included in analyses.

In this study, we investigated the genotype of the AT1 gene in patients with HP and normal pregnant women and detected a significant association of the C1166 variant of the AT1 gene with HP. Further, we carried out a multivariate analysis of the AT1 and AGT genotypes in primiparous subjects and found that these were independently associated with HP, suggesting that the C1166 variant of the AT1 gene may be an additional risk factor for HP.

Materials and methods

Subjects

Japanese patients with severe HP and controls were recruited from women who delivered singletons at Hokkaido University Hospital and its affiliated hospitals. Women with preexisting hypertension were excluded to make the present cases the pure type of HP. Women with renal disease, diabetes mellitus, amniotic volume abnormalities, or fetal anomalies were also excluded. Severe HP was diagnosed according to the criteria of the National High Blood Pressure Education Program Working Group (1990). The criteria

for severe HP were as follows: (1) a blood pressure reading of 160/110 mmHg or more after the 20th gestational week, (2) proteinuria was defined as the excretion of 30 mg/dl (1+ on dipstick) or greater, (3) women with blood pressure of more than 140/90 mmHg or proteinuria prior to the 20th gestational week or 4 weeks after delivery were excluded from the HP subjects because they may have had latent hypertensive or renal diseases. Finally, 114 patients with severe HP agreed to participate in this study between 1993 and 1996, and 291 controls were randomly selected from normal pregnant women who also agreed to participate in the study between April 1993 and March 1994. Informed consent for the study was obtained from every subject following guidelines for informed consent in epidemiological studies in Japan (Tamakoshi et al. 2000), and the present study was approved by the institutional review board of Hokkaido University School of Medicine.

Clinical characteristics and pregnancy outcomes of cases of severe HP and controls are shown in Table 1. Prepregnancy body mass index (BMI) and the rate of positive family history of hypertension were significantly higher in the women with severe HP than in the controls, while no significant differences in the rate of primiparas or the maternal age were detected between the two groups. The gestational weeks at delivery were higher and birth weight significantly lower in the severe HP group than in the controls. About 30% of severe HP cases were complicated with intrauterine fetal growth restriction.

Genotyping

Genomic DNA was extracted from peripheral leukocytes and the genotyping with respect to the A1166C variant of the AT1 gene was performed by using allele-specific oligonucleotide hybridization of PCR-amplified products as previously described (Bonnardeaux et al. 1994). The occurrence of T235 homozygotes (TT) of the AGT gene was also examined by using T *th*1111 digestion of PCR-amplified products as previously described (Russ et al. 1993).

Statistical analysis

Statistical analyses were performed to compare the number of C1166 alleles with that of A1166 alleles for the AT1 gene and the number of C1166 homozygous (CC) plus heterozygous (AC) women with that of A1166 homozygous (AA) women. The differences were analyzed statistically by the chi-square test (df=1). Yates' correction for continuity was used when an observed number was ≤ 5 .

In the multivariate analysis, a stepwise method was applied to select significant ($p < 0.05$) factors among four factors existing before pregnancy that were significantly associated with HP in univariate analyses, i.e., "family history of hypertension," "prepregnancy high body mass index (BMI ≥ 25)," "T235 homozygous

Table 1 Clinical characteristics and pregnancy outcomes of women with severe hypertension in pregnancy and normal controls

	Parity	Severe hypertension in pregnancy (n = 114)	Controls (n = 291)
Percentage (number) of primiparas		64.0 (73/114)	56.7 (165/291)
Maternal age (years) (mean \pm SE)		29.7 \pm 0.5	29.2 \pm 0.3
	Primipara	27.7 \pm 0.5	27.3 \pm 0.3
Family history of hypertension (%)		30.8**	13.1
	Primipara	31.5**	15.2
Prepregnancy body mass index (mean \pm SE)		22.5 \pm 0.4*	21.1 \pm 0.2
	Primipara	21.7 \pm 0.4	20.7 \pm 0.2
Gestational weeks at delivery (mean \pm SE)		36.6 \pm 0.3**	39.1 \pm 0.1
	Primipara	37.0 \pm 0.3**	39.2 \pm 0.1
Birth weight of neonates (g) (mean \pm SE)		2,444 \pm 80**	3,160 \pm 21
	Primipara	2,478 \pm 94**	3,122 \pm 27
Percentage of intrauterine growth restriction (%)		31.6	0
	Primipara	37.0	0

* $p < 0.01$, ** $p < 0.001$ versus controls

(TT) genotype of AGT gene," and "AC+CC genotype of AT1 gene," adjusted for "maternal age." A multiple logistic model was applied to evaluate the odds ratios of the major risk factors. All statistical analyses were conducted by use of a statistical analysis system package (SAS Institute, Inc., Cary, NC, USA).

Results

Table 2 shows the distribution of A1166C variants of AT1 determined in 114 patients with severe HP (73 primiparous and 41 multiparous women) and 291 normal controls (165 primiparous and 126 multiparous women). Among the total subjects, the frequencies of allele C and women with AC+CC genotypes were significantly higher in severe HP (10.1 and 18.0%) cases than in controls (5.8 and 10.8%, respectively) (both $p < 0.05$). Among primiparous subjects, the frequencies were also significantly higher in severe HP (10.3 and 19.2%) cases than in controls (4.2 and 7.9%, respectively) (both $p < 0.05$), whereas no significant differences were found among multiparous subjects (9.8 and 17.1% in severe HP vs 7.9 and 14.3% in controls, respectively).

Table 3 shows the odds ratios of selected risk factors before pregnancy for primiparous severe HP. In univariate analyses, four factors, i.e., "AC+CC genotype of AT1," "TT genotype of AGT," "prepregnancy BMI ≥ 25 ," and "family history of hypertension" were significantly associated with primiparous severe HP. Further, multivariate analysis to clarify genotype-genotype confounding with "AC+CC genotype of AT1" and "TT genotype of AGT" revealed that they were independently associated with primiparous severe HP. When "family history of hypertension" and "prepregnancy

BMI ≥ 25 " were added as examined factors for further multivariate analysis, "TT genotype of AGT" and "family history of hypertension" were found to be independent potent factors. The odds ratios of the two factors were 2.7 and 2.2, respectively, in a multiple logistic model.

Discussion

The association between C1166 of the AT1 gene and HP was reported in Caucasian (Nalogowska-Glosnicka et al. 2000) and Polish (Seremak-Mrozikiewicz et al. 2000) subjects. The present study demonstrated not only the association of the AT1 variant with severe HP but also interrelationships among the variants of the AT1 and the AGT genes, high BMI, and family history of hypertension in the manifestation of severe HP in Japanese subjects. The distribution of the AT1 variants in controls observed in this study was compatible with that found in the Japanese population at large (Ono et al. 2003) and displayed Hardy-Weinberg equilibrium.

The category of HP contained two subtypes: women with proteinuric and/or edematous type HP (PE) and women without those features (gestational hypertension; GH). Regarding PE, villous disorders, including abnormal pregnancy-induced spiral artery remodeling occurring early in pregnancy, are thought to be among its causes (Morgan et al. 1999), whereas most GH is believed to be latent essential hypertension unmasked during pregnancy (National High Blood Pressure Education Program Working Group 1990). In the present study, the frequencies of C1166 and AC+CC genotypes

Table 2 Distribution of A1166C variants of AT1 in women with severe hypertension in pregnancy and controls

Parity	Diagnosis	No. of cases studied	Genotype			Frequency (%)	
			AA	AC	CC	Allele C	AC+CC
Primipara	SHP	73	59	13	1	10.3*	19.2*
	Controls	165	152	12	1	4.2	7.9
Multipara	SHP	41	34	6	1	9.8	17.1
	Controls	126	108	16	2	7.9	14.3
Total	SHP	114	93	19	2	10.1*	18.0*
	Controls	291	260	28	3	5.8	10.8

SHP, severe hypertension in pregnancy

* $p < 0.05$ vs controls tested by chi-square test (df = 1)

Table 3 Odds ratios of selected risk factors existing before pregnancy for primiparous severe hypertension in pregnancy: multivariate analysis

	Crude OR (95%CI)	OR (95%CI) ^a	OR (95%CI) ^b
AC+CC genotype of AT1	2.8 (1.2-6.3)*	2.4 (1.0-5.7)*	2.3 (1.0-5.6)
TT genotype of AGT	2.6 (1.4-4.8)**	2.7 (1.4-5.1)**	2.7 (1.4-5.4)**
Pregpregnancy BMI ≥ 25	2.5 (1.0-6.1)*	-	2.6 (1.0-6.7)
Family history of hypertension	2.6 (1.3-4.9)**	-	2.2 (1.1-4.5)*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

^aMultivariate analysis with AC+CC genotype of AT1 and TT genotype of AGT, adjusted for maternal age

^bMultivariate analysis with AC+CC genotype of AT1, TT genotype of AGT, prepregnancy BMI ≥ 25 and family history of hypertension, adjusted for maternal age

were 19.6 and 34.7% (8/23), respectively, in severe GH and 7.7 and 14.2% (13/91), respectively, in severe PE (the data are not shown in the tables). Our results seem to be consistent with the previous reports that demonstrated an association between C1166 of the AT1 gene and essential hypertension (Bonnardeaux et al. 1994; Szombathy et al. 1998; Jiang et al. 2001), although the number of the cases are small yet. We think it is necessary in future studies to clarify whether C1166 is particularly associated with severe GH or not and whether C1166 is associated with cases that have both GH and latent essential hypertension.

In the final multivariate analysis, the factors "family history of hypertension" and "prepregnancy BMI ≥ 25 " reduced the *p*-values of "AC+CC genotype of AT1," and eliminated the significance, suggesting that these factors were not independent of each other, whereas, "TT genotype of AGT" was found to be independent of these factors. This result was consistent with the previously proposed hypothesis that the T235 variant of the AGT gene may be concerned with abnormal pregnancy-induced spiral artery remodeling (Morgan et al. 1999). On the other hand, the AC(CC) genotypes of AT1 may be related to the family history of hypertension and prepregnancy high BMI, which were concerned with the predisposition to essential hypertension.

The molecular and biochemical mechanism by which the A1166C variant of the AT1 gene is involved in the manifestation of HP is still obscure, since the variable nucleotide is located in the 3' untranslated region (Duncan et al. 2001). The allele C1166 has been reported to be associated with aortic stiffness (Lajemi et al. 2001) but not associated with hypertension in the elderly (Liyou et al. 1999). In the present study, no significant association was found between AT1 and HP in elderly pregnancy, and multivariate analysis also revealed that the association of AT1 with HP was independent of maternal age. It was reported that the A1166C polymorphism does not have a major effect on the actions of angiotensin II (Hilgers et al. 1999), while the polymorphism was found to be associated with salt sensitivity in hypertensive patients (Spiering et al. 2000). Elucidation of whether there is synergism and/or interaction between the AT1 genotype and dietary salt intake during pregnancy in the manifestation of HP will be the next goal of our future studies.

In summary, C1166 of the AT1 gene was associated with severe HP independently of T235 of the AGT gene in Japanese subjects. Further studies enrolling larger population samples and different ethnic groups will be useful for elucidating the pathogenesis of HP and establishing preventive strategies against HP.

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ARTICLE

CD40 ligand gene and Kawasaki disease

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Kawasaki disease (KD) is an acute systemic vasculitis syndrome of infants and young children. Although its etiology is largely unknown, epidemiological findings suggest that genetic factors play a role in the pathogenesis of KD. To identify genetic factors, affected sib-pair analysis has been performed. One of the identified peaks was located on the Xq26 region. A recent report of elevated expression of CD40 ligand (CD40L), which maps to Xq26, during the acute-phase KD, and its relationship to the development of coronary artery lesions (CAL) prompted us to screen for polymorphism of CD40L and to study the association of the gene to KD. A newly identified SNP in intron 4 (IVS4 + 121 A > G) is marginally over-represented in KD patients as compared to controls (109/602, 18.1 vs 111/737, 15.1%). When male KD patients with CAL were analyzed as a patient group, the SNP was significantly more frequent than in controls (15/58, 25.9%, vs 111/737, 15.1%, OR = 2.0, 95% CI = 1.07–3.66; $P = 0.030$). Interestingly, this variation was extremely rare in a control Caucasian population (1/145, 0.7%). Our results suggest a role of CD40L in the pathogenesis of CAL and might explain the excess of males affected with KD.

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Introduction

Kawasaki disease (KD) is an acute systemic vasculitis of young children characterized by mucous membrane inflammation, cervical lymph adenopathy, rash and fever.¹ It

mainly affects children younger than 5 years of age and the peak of the onset is near 6 months. Although it is usually a self-limited disorder, serious, and sometimes life-threatening complications related to coronary artery aneurysms can develop in 15–20% of untreated patients.² The disease is the leading cause of acquired heart diseases in Japan and also in the US.³ Since Kawasaki¹ described the disease in 1967, extensive efforts have been made to identify the etiology of the disease. However, to date, the etiology still remains elusive. Genetic factors are suspected to influence the susceptibility to an unknown hypothetical pathogen(s) based on the following two epidemiological studies. First, siblings and offspring of KD patients are at higher risk for the disease.^{4,5} Second, marked ethnic differences in KD susceptibility exist with increased prevalence of KD among Japanese Americans in Hawaii.⁶ The human genome project has provided us with new tools for examining genetic susceptibility to disease. Almost 200 000 well-characterized single-nucleotide polymorphisms (SNPs) for the entire genome of the Japanese population are now available (IMS JSNP database, <http://snp.ims.u-tokyo.ac.jp/>). Genome-wide microsatellite information is also available (UniSTS, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists>). With these resources, we decided to search for genes for KD susceptibility using a genome-wide scan. Initially, we performed affected sib-pair linkage analysis and have identified four possible loci, 12q24, 7p15, 19q13.3, and Xq26 (Onouchi *et al*, manuscript in preparation). The fact that male children are predominantly affected with KD (male/female = 1.4)⁷ suggests that a gene on the X chromosome could be implicated in KD susceptibility.

Recently, elevated expression of CD40 ligand (CD40L) on CD4+ helper T cells and platelets during the acute-phase KD and significantly higher expression in KD patients with coronary artery lesions (CAL) was reported.⁸ We found that the CD40L gene locates on Xq26, the same region of identified peak by our linkage analysis. In addition, CD40L is known to induce endothelial cells to produce cell adhesion molecules and chemokines, and these molecules are suspected to play important roles in the pathophysiology of KD.^{9–15} Thus, we hypothesized that *CD40L* is a strong candidate gene whose genetic alteration could influence KD susceptibility. To test this hypothesis, we performed the following experiments. First, the entire 12.2 kb gene region containing 3.4 kb 5' upstream was sequenced to find SNPs. We identified a total of 22 SNPs, four in the 5' upstream region, one in exon 1, one in intron 1, one in intron 2, 10 in intron 4 and five in exon 5. In all, 10 of the 22 SNPs were novel. A CA dinucleotide repeat in the 3' untranslated region of exon 5 was also used as a marker. Second, an association study of these polymorphisms with 427 Japanese KD patients and 476 healthy Japanese controls was conducted. Among these polymorphisms, a novel SNP in intron 4, which is

marginally over-represented in KD altogether, has revealed to be significantly more frequent in male KD patients with CAL when compared to controls. Our findings may suggest a role of CD40L in the pathogenesis or disease severity of KD. Considering an effect of lyonization on the X chromosome genes and the extremely rare frequency of this SNP in Caucasian population (0.7%), this polymorphism is a good candidate to explain the sex and/or ethnic differences in KD susceptibility and outcome.

Materials and methods

Patients and samples

All patients were diagnosed with KD by pediatricians according to the criteria established by the Japan Kawasaki Disease Research Committee (<http://www.kawasaki-disease.org/diagnostic/index.html>). CAL was diagnosed with dilated coronary artery lesions measured by two-dimensional echocardiography under the definition in the guideline.¹⁶ Of 427 patients studied, 81 were the probands of sib-pair or sib-trio cases and the remaining were sporadic cases. In total, 476 Japanese volunteers constituted by 338 healthy adults without history of KD and 138 children visiting the hospitals to be treated for their illness other than KD were selected as controls. The Ethical Committee of RIKEN approved this study, and all patients and Japanese volunteers gave written informed consent for blood sampling and DNA analysis. EB virus-transformed lymphoblastoid cell lines were established in 42 of the sibling cases using previously described methods.¹⁷ Genomic DNA was extracted from peripheral leukocytes or such cell lines by standard procedures.¹⁸ In all, 96 DNA samples of unrelated Caucasian individuals were purchased from the Coriell Institute for Medical Research.

Polymerase chain reaction

All polymerase chain reactions (PCRs) were performed on GeneAmp 9700 thermal cyclers (Applied Biosystems, Foster City CA, USA). PCR thermocycling parameters used in discovery and genotyping SNPs were first denatured for 5 min at 94°C, followed by 37 cycles of 30 s each at 94, 60 and 72°C, and a final extension step of 7 min at 72°C.

Discovery of variations

Approximately 16 kb of genomic region containing all exons, introns and 3.4 kb of 5'-flanking of the *CD40L* gene was sequenced in search of variations. Repetitive sequences were masked using the RepeatMasker program (<http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker>) and excluded from the screening. PCR amplification was performed in a 16- μ l reaction volume containing 67 mM Tris-HCl, 16.6 mM (NH₄)₂SO₄, 6.7 mM MgCl₂, 10 mM β -mercaptoethanol, 6.7 mM EDTA, 1.5 mM of each dNTP, 0.45 mM of each primer, 1.0 U of Ex Taq™ Hot Start Version (Takara, Tokyo, Japan) and 10 ng of genomic DNA.

DNA samples from unrelated 24 females, which included 18 KD patients and six healthy controls were analyzed. Sequences, positions and sizes of amplicons of the each PCR primer pair used were summarized (Supplementary Table 1). Purified and diluted PCR products were sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits v2.0 (Applied Biosystems, Foster City CA, USA). Electrophoresis was conducted on an ABI 3700 auto sequencer (Applied Biosystems, Foster City CA, USA). GAP4 program contained in Staden Package (<http://www.mrc-lmb.cam.ac.uk/pubseq/>) was used to detect variations.

Linkage disequilibrium analysis

Pairwise LD analysis for 22 SNPs discovered in the screening was performed using the SNPalyze V3.0. program (Dynacom, Mobara, Japan). Tightly linked ($r^2 > 0.5$) SNPs were grouped and representative SNPs from each group were analyzed in the subsequent case-control study.

Genotyping of SNPs

Genotyping of the SNPs was carried out by PCR-RFLP, except for SNP140. Mismatch primers designed to generate restriction enzyme recognition sequences including SNPs within them were used in each primer pair. After PCR amplification, products were digested using the appropriate restriction enzyme according to the manufacturers' instructions. Primers and restriction enzymes used are listed in Supplementary Table 2. Separation was performed on 4% agarose gels in TBE buffer. The gels were stained by ethidium bromide. Direct sequencing of PCR fragments was conducted for genotyping SNP140. Primers used to amplify 357bp of the SNP140 region were 5'-TCTTTGCGTGTCAGTGTCTTCC-3' and 5'-TGTTAGAAA GGGGATTGAGAAG-3'.

Genotyping of dinucleotide repeats

PCR amplification of dinucleotide repeat located in the last exon of *CD40L* gene was performed using the following primers (forward primer 5'-AGTCTCTTCCCTCCCCA GTCT-3'; reverse primer 5'-GAACTGACTAGCAACGGCCT

GA-3'). The forward primer was labeled with VIC (Applied Biosystems, Foster City CA, USA). Amplified samples were diluted and mixed with deionized formamide containing LIZ-labeled molecular size marker (GeneScan 500LIZ, Applied Biosystems, Foster City, CA, USA). Electrophoresis was conducted on an ABI 3700 auto sequencer (Applied Biosystems, Foster City CA, USA). Analyses of the electropherogram results and size calling of amplified DNA fragments was performed using Genescan 3.5.2 software and Genotyper 3.7 software (Applied Biosystems, Foster City CA, USA). In all, 473 healthy Japanese individuals (210 males and 263 females), 81 probands of sib-pair cases (40 males and 41 females) and 308 sporadic cases of KD (191 males and 117 females) were genotyped. Subsequently, estimation of allele frequency and analyses of linkage and association were performed.

Statistical analysis

Frequencies of alleles and genotypes in KD cases and controls were compared using χ^2 tests. Two-point sib-pair analysis for dinucleotide repeat was conducted by using MAPMAKER/SIBS program version 2.1 (<ftp://ftp-genome.wi.mit.edu/distribution/software/sibs>) in the sex-linked mode. In this method, brother-brother, brother-sister and sister-sister sib pairs are separately analyzed.¹⁹ It is the extended Holman's method in which the maximization is restricted to the following genetically valid values: $0 \leq z_{1bb} \leq 0.5$, $0 \leq z_{1bs} \leq 0.5$, $0 \leq z_{1ss} \leq 0.5$. z_1 represents the probability of IBD sharing of maternal allele.²⁰

Results

Identification of variations in *CD40L*

A total of 22 SNPs were identified by sequencing the entire *CD40L* gene (12.2 kb), including the 3.4 kb 5' upstream region that includes the previously described promoter,²¹ and all five exons and introns (Figure 1 and Table 1). In all, 12 of the 22 SNPs were identical to those in the dbSNP database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp>). The remaining 10 SNPs were located as follows: SNP95 in the 5' flanking region, SNP02 in intron

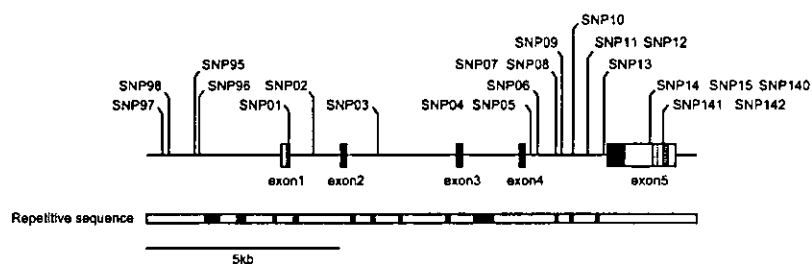


Figure 1 Genomic organization of *CD40L*. Locations of SNPs in the *CD40L* gene. Open and filled rectangles represent untranslated and coding regions, respectively. A CU-rich element and a CA dinucleotide repeat (XqM5257) in exon 5 are represented by rectangles, dotted and striped respectively. Location of repetitive elements is indicated in a horizontal bar below exon numbers as blackened zones.

Table 1 Identified SNPs in the CD40L gene

SNPID	Location	Position	5'-flanking sequence	SNPs	3'-flanking sequence	Substitution	dbSNP number
SNP97	5'-flanking region	-3419 ^a	gagaattagccttgccacac	A>G	aataacatgaataagcacag		rs3092952
SNP98	5'-flanking region	-3183 ^a	gttatatttaactctttcat	A>G	tgactggaaatgatttcc		rs3092951
SNP95	5'-flanking region	-2773 ^a	ttcccttgatattgctaca	C>A	atcaatcactagctagcaga		
SNP96	5'-flanking region	-2608 ^a	agagaaagagaaggctcaa	C>G	aattgaggtaagcagggc		rs3092948
SNP01	Exon 1	187 ^a	ctgtgatcttcataagaagg	T>C	tggacaaggaagatgaacc	Leu 63 Leu	rs1126535
SNP02	Intron 1	IVS1+725	catcaggacttatttggttt	T>C	catgtatcttctcactcca		
SNP03	Intron 2	IVS2+1171	tacactaatctaggccttag	A>G	tgaactcctaaacccttc		rs3092943
SNP04	Intron 4	IVS4+121	caatttacaacactaccct	A>G	cacttctcctattccctct		
SNP05	Intron 4	IVS4+171	aagtgagttcaaatgcacag	A>G	tgggacttagagggacaaaa		
SNP06	Intron 4	IVS4+346	agcatccctcctcctaacc	A>C	tctcactgtgtggaaatgg		rs3092929
SNP07	Intron 4	IVS4+517	tccaatgaagtaacaacaga	C>T	gacatattgtgatctttca		
SNP08	Intron 4	IVS4+685	atgcacgctaacataaagtc	T>C	cccatcaacataaaggcaag		rs3092928
SNP09	Intron 4	IVS4+801	tatgtgatgtgtgtgacag	G>A	gagagaagagagatggaca		rs3092927
SNP10	Intron 4	IVS4+1095	tggccgaaagtgtgtgtgtc	G>A	agtgtgtacaaccgtaagg		rs3092926
SNP11	Intron 4	IVS4-926	cctccctattagtaaaagggc	G>A	agtcttaccaggtggatct		rs3092925
SNP12	Intron 4	IVS4-752	tggcagctgagatttcaagt	C>T	gggtgcacacactacttga		rs3092924
SNP13	Intron 4	IVS4-13	cctcaccacaactttccct	T>C	Tcttgaacagtgttacag		rs3092923
SNP14	3'-untranslated region	11714 ^a	cacagtgagaaaccgaaacc	C>G	Ccccccccccccgccacc		
SNP140	3'-untranslated region	11725 ^a	accgaaacccccccccccc	C>T	Ccgccaccctctcgacagt		
SNP15	3'-untranslated region	11739 ^a	cccccccccgccaccctctc	G>A	Gacagttattcattctctt		
SNP141	3'-untranslated region	11908 ^a	cccttctaacacacacaca	C>T	Acacacacacacacacac		
SNP142	3'-untranslated region	11910 ^a	ctttctaacacacacacaca	C>A	Acacacacacacacacac		

^aDistance from the transcriptional start site.

Table 2 Allele frequencies of SNPs of the CD40L gene in KD patients and controls

SNPs	Male		Female		All	
	KD	Control	KD	Control	KD	Control
SNP95	8/251 (3.2)	12/212 (5.7)	12/346 (3.5)	17/524 (3.2)	20/597 (3.4)	29/736 (3.9)
SNP01	33/250 (13.2)	18/213 (8.5)	34/348 (9.8)	59/524 (11.3)	67/598 (11.2)	77/737 (10.4)
SNP02	8/253 (3.2)	4/211 (1.9)	4/348 (1.2)	13/518 (2.5)	12/601 (2.0)	17/729 (2.3)
SNP03	0/251 (0.0)	0/212 (0.0)	1/346 (0.3)	0/524 (0.0)	1/597 (0.2)	0/736 (0.0)
SNP04	43/253 (17.0)	41/213 (19.2)	66/348 (19.0)	70/524 (13.4)	109/601 (18.1)	111/737 (15.1)
SNP07	5/251 (2.0)	3/213 (1.4)	3/348 (0.9)	6/524 (1.1)	8/599 (1.3)	9/737 (1.2)
SNP08	5/251 (2.0)	3/213 (1.0)	10/348 (2.9)	15/524 (2.9)	15/599 (2.5)	18/737 (2.4)
SNP140	0/176 (0.0)	1/103 (1.0)	5/274 (1.8)	3/348 (0.9)	5/450 (1.1)	4/451 (0.9)

Two numerals divided by '/' represent frequencies of minor alleles and total chromosomes genotyped, respectively, and the percentages are shown in parentheses.

1, SNP04, 05 and 07 in intron 4 and SNP14, 15, 140, 141 and 142 in exon 5. All but one SNP in exon 1, which is synonymous, were located outside the coding region (Table 1). Three SNPs in exon 5, SNP14, 15 and 140, were in close proximity to the previously described CU-rich element,^{22,23} where members of the polypyrimidine tract-binding proteins (PTB) family bind and play a role in regulating CD40L expression at the level of post-transcriptional mRNA decay (Figure 1). The possible existence of rare deleterious mutations or polymorphisms altering translation of the protein has been ruled out by re-sequencing all five exons of CD40L in DNA derived from 81 probands of sibling cases.

Linkage disequilibrium (LD) analysis

To evaluate the relationship among these 22 SNPs, pairwise LD analysis was performed. LD coefficients based on the

genotype frequencies of 24 female subjects were shown (Supplementary Table 3). The |D'| statistics were all 1.0. Strong LD was observed in the following two groups of SNPs (group 1: SNP97, 98, 96, 1, 5, 6, 9 and 13; group 2: SNP08, 10, 11, 12, 14, 15, 141 and 142), with the r^2 statistics >0.5 between every two SNPs in each group. Thus, one SNP in each group was selected for genotyping and further analysis.

Case-control study

SNP01 and 08 were chosen as representatives of the two LD groups for genotyping in the case-control study. Six SNPs (SNP95, 02, 03, 04, 07 and 140) that did not belong to the LD groups were also genotyped (Table 2). No significant difference was observed in the allele frequency of these SNPs between KD patients and healthy controls. However, in a stratified analysis using sex and coronary artery status,

Table 3 Allele frequencies of IVS4+121 A>G polymorphism of CD40L gene in male KD patients with or without CAL, Japanese controls and Caucasians

	A	(%)	G	(%)	OR (95% CI)	P
Male KD with CAL	43	(74.1)	15	(25.9)		
Male KD without CAL	167	(85.6)	28	(14.4)	2.1 (1.02–4.21) ^a	0.041
Control	626	(84.9)	111	(15.1)	2.0 (1.07–3.66) ^a	0.030
Caucasian	144	(99.3)	1	(0.7)		

^aCompared to male KD with CAL.

Table 4 Sib-pair analysis of a CA dinucleotide repeat

	n	z ₀	z ₁	LOD
Brother/brother pairs	25	0.28	0.72	0.71
Brother/sister pairs	37	0.50	0.50	0.00
Sister/sister pairs	18	0.33	0.67	0.30
Total	80			1.01

z₀ and z₁ represent the estimated proportion of sib pairs sharing, 0 and 1 maternal allele identical by descent, respectively.

male patients with CAL had an increased frequency of the minor allele of SNP04 (IVS4 + 121 A>G) as compared to the control group (25.9 vs 15.1%; OR = 2.0, 95% CI = 1.07–3.66, $\chi^2 = 4.70$, $P = 0.030$). While comparison between male patients with CAL and those without CAL also showed a significant difference (25.9 vs 14.4%; OR = 2.1, 95% CI = 1.02–4.21, $P = 0.041$) (Table 3), no difference was observed between female patients with CAL and those without CAL (data not shown). To estimate the allele frequency in a Caucasian population, 96 samples (47 males and 49 females; Coriell Institute, Camden, NJ) were genotyped. Only one allele in 145 X chromosomes carried the G allele (0.7%) (Table 3).

Analyses of dinucleotide repeat in 3'UTR

A polymorphic dinucleotide repeat sequence (XqMS257) locates in the 3'-UTR of the CD40L gene (Figure 1). We tested the relevance of the dinucleotide repeat by two-point sib-pair analysis. There is no difference in allele frequencies of the polymorphic sequence between 389 KD patients and 473 healthy controls (Supplementary Table 4). However, we observed excess sharing of alleles and nominal evidence of linkage in 80 KD sibling cases (LOD score = 1.0) (Table 4).

Discussion

KD is an acute systemic vasculitis syndrome of unknown etiology. Three KD epidemics of 1979, 1982 and 1986 in Japan suggest the role of unidentified infectious agent(s) in

the pathogenesis of the disease. In addition, genetic factors influencing susceptibility to KD have been postulated.^{4–6} In our genome-wide sib-pair analysis, Xq26, where CD40L mapped, is one of the loci that showed linkage (Onouchi *et al*, manuscript in preparation). CD40L is a type II integral membrane protein and belongs to the tumor necrosis factor (TNF) gene family. The protein is expressed on the surface of activated CD4+ T cells and platelets and interacts as a ligand of CD40 molecule expressed on various types of cells. It is well known that CD40L plays an important role in host defense, regulation of autoimmunity and tumor growth.⁹ Deleterious mutations in CD40L cause X-linked hyperimmunoglobulin M syndrome (HIGM1).^{24–26} HIGM1 is characterized by elevated serum IgM in contrast to the absence of IgG, IgA and IgE, and patients with this disorder have increased susceptibility to bacterial infections. Thus, variations modulating function or quantity of the CD40L protein could be postulated to the response to infectious agents. Indeed, an SNP in the 5' flanking region of *CD40L* was associated with reduction in risk for severe malaria.²⁷ KD is thought to develop as a result of excessive activation of the immune system triggered by some infectious events. Wang *et al*⁸ reported that CD40L expression on T cells and platelets was significantly higher in acute KD patients as compared to febrile controls. Moreover, in KD patients, the expression level of CD40L on CD4+ T cells and platelets is correlated with CAL.⁸ On the other hand, CD40L expressed on T cells and platelets is known to interact with CD40 on endothelial cells and induce them to express cell adhesion molecules such as E-selectin, ICAM-1 and VCAM-1¹⁰ and secrete IL-8 and MCP-1.¹¹ In an acute phase of KD, it is known that these molecules are upregulated and thought to play important roles in the pathogenesis of the disease.^{12–15} Therefore, CD40L is a candidate gene relevant to KD susceptibility.

In the present study, we identified 22 SNPs of the *CD40L* gene in the Japanese population. Although this gene region was known as an 'SNP desert'²⁸ with low SNP incidence, and was reported to have fewer SNPs in Asian,²⁹ a relatively large number of SNPs were identified in this study. SNP01, the only SNP within the protein-coding region, was synonymous and coded as rs1126535 in the

dbSNP database. The allele frequency of the SNP in the present Japanese population was 10.5%, about half that reported in the database (22.5%). Among SNPs identified in this study, the minor allele frequency of SNP04, which was associated with CAL in male patients, was the highest in the Japanese. In contrast, allele frequency was only 0.7% in Caucasians. The Japanese nationwide surveys of KD incidence have documented that male children are more often affected and have a higher risk for cardiac complications.⁷ Unlike adults, no differences in lifestyle or environment are conceivable in early childhood. Thus, it seems rational to postulate that one of the genes influencing KD susceptibility and disease severity is located on the X chromosome. Although it is clear that larger numbers of patients with CAL will need to be analyzed to obtain a conclusive result, the finding that SNP04 is very rare in Caucasians is interesting when the low incidence rate of KD is considered. It is unknown whether this SNP by itself affects the transcription, splicing or stability of CD40L mRNA. It is possible that the observed association with SNP04 is due to a genetic variation at a different locus that is in linkage disequilibrium with this marker.

CU-rich *cis*-acting elements known to regulate mRNA stability^{22,23} exist in the 3'-UTR of the gene. Although a total of five SNPs were identified in the 3'-UTR (Figure 1), none of these polymorphisms are located within the element. In the 5'-flanking region, there are three NFAT transcription factor-binding sites, the most distal one being about 760 bp upstream of the transcription start site.²¹ Again, no variation likely to be involved in transcriptional factor binding was identified despite extensive screening.

Eight haplotypes were determined by genotyping seven SNPs in male subjects. A CA repeat near the CU-rich element was found to be highly polymorphic. Although no positive association was found with the haplotypes and the CA repeat, the new information about polymorphisms in this study provide a valuable tool for the future studies of other diseases. Validation of our current findings in larger cohorts of KD patients will define the importance of CD40L SNP04 in disease susceptibility and outcome.

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Association analysis of *SLC22A4*, *SLC22A5* and *DLG5* in Japanese patients with Crohn disease

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Abstract Crohn disease (CD) is an inflammatory bowel disease characterized by chronic transmural, segmental, and typically granulomatous inflammation of the gut. Recently, two novel candidate gene loci associated with CD, *SLC22A4* and *SLC22A5* on chromosome 5 known as *IBD5* and *DLG5* on chromosome 10, were identified through association analysis of Caucasian CD patients. We validated these candidate genes in Japanese patients with CD and found a weak but possible association with both *SLC22A4* ($P=0.028$) and *DLG5* ($P=0.023$). However, the reported genetic variants that were indicated to be causative in the Caucasian population were completely absent in or were not associated with Japanese CD patients. These findings imply significant differences in genetic background with CD susceptibility among different ethnic groups and further indicate some difficulty of population-based studies.

Keywords Crohn disease · Single-nucleotide polymorphism (SNP) · *DLG5* · *SLC22A4* · *SLC22A5* · *OCTN1* · *OCTN2* · Japanese population

Introduction

Inflammatory bowel diseases (IBDs), which are usually classified into two clinical entities—Crohn disease (CD; MIM 266600) and ulcerative colitis (UC)—are chronic conditions characterized by remitting and relapsing inflammation of the small and/or large intestines. Familial aggregation and twin studies indicate a presence of genetic factors susceptible to this condition. Genome-wide linkage analyses have localized genes conferring susceptibility to IBD to several possible candidate loci on chromosomes 1, 3, 5, 6, 7, 10, 12, 14, 16, 19, and 22 (Hugot et al. 1996; Satsangi et al. 1996; Cho et al. 1998; Duerr et al. 2000; Hampe et al. 1999; Rioux et al. 2000, 2001).

Among the several candidate loci, susceptible genes at two distinct loci were recently identified through the evidences of strong association with the CD phenotype. In the *IBD5* locus on chromosome 5, single-nucleotide polymorphisms (SNPs) in two candidate genes, *SLC22A4* and *SLC22A5*, both of which encode organic cation transporters, revealed significant associations with CD (Peltekova et al. 2004). A C1672T substitution in exon 9 of the *SLC22A4* gene and a G-207C in the *SLC22A5* promoter region were indicated as functional and causative mutations to increase susceptibility to CD. The other gene identified from chromosome 10 was the *DLG5* gene, encoding a scaffolding protein involved in the maintenance of epithelial integrity. Risk-associated variants, including a G113A substitution in exon 3 of the *DLG5* gene, constructed two distinct haplotypes with a replicable distortion in transmission (Stoll et al. 2004).

To investigate a possible role of these candidate gene loci, one corresponding to *SLC22A4* and *SLC22A5* and another corresponding to *DLG5*, in the pathogenesis of

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CD in Japanese, we examined SNPs of these three genes in a large number of clinical samples. We here report an absence of DNA substitutions or lack of association for the candidate-causative SNPs, which were indicated in the previous reports, in the Japanese CD patients. However, we observed a weak association of other genetic substitutions in these genes of Japanese patients with CD. Our results indicate that the reported substitutions in the three genes are unlikely to be causative to Japanese CD patients, but the candidacy of these two loci for Japanese CD cannot be totally excluded.

Materials and methods

Subjects and DNAs

Japanese blood samples were obtained with written informed consent from 484 CD patients at the Social Insurance Central General Hospital and from 345 unaffected control individuals belonging to the Osaka-Midosuji Rotary Club. All CD cases were diagnosed at the Inflammatory Bowel Unit of the Social Insurance Hospital by clinical, radiological, endoscopic, and histological findings according to the Lennard-Jones' criteria (Lennard-Jones 1989). Patients with indeterminate colitis were excluded. DNAs were prepared from these samples according to standard protocols.

DNA sequencing

To search genetic variations in these candidate loci including the five reported variants, C1672T in exon 9 of *SLC22A4* and G-207C in the *SLC22A5* promoter, as well as G113A in exon 3, C4136A in exon 23 and 35delA in intron 26 of *DLG5*, we carried out direct sequencing of those regions in 48 individuals with confirmed diagnosis of CD by means of the BigDye Terminator RR Mix (Applied Biosystems, USA) with ABI 3700 sequencers using the primers listed in Table 1.

Markers

SNPs in the *SLC22A4* and *SLC22A5* genes were screened according to methods described previously (Saito et al. 2002). We selected 17 SNPs, including six in *SLC22A4* (*SLC22A4* 1–6), three in *SLC22A5* (*SLC22A5* 1–3), and eight in *DLG5* (*DLG5* 1–8) (Table 2). Information for each SNP in the *SLC22A4*, *SLC22A5*, and *DLG5* chosen for this study was obtained from the Japanese SNP (JSNP) database (<http://snp.ims.u-to-kyo.ac.jp>) (Hirakawa et al. 2002; Haga et al. 2002).

SNP analysis and genotyping

We amplified multiple genomic fragments using 20 ng of genomic DNA for each polymerase chain reaction (PCR), as described previously (Ohnishi et al. 2001). We genotyped all participants for a total of 17 SNPs indicated in Table 2 by means of the Invader assay (Mein et al. 2000). The C4136A in exon 23 and 35delA in intron 26 (deletion of an adenine at the 35th nucleotide in intron 26) of *DLG5* were examined by direct sequencing using the same primers.

Statistical analysis

Genotype distributions and allele frequencies of each SNP were compared, respectively, between the cases and the controls, as described elsewhere (Yamada et al. 2001). Haplotype frequencies were estimated using the expectation-maximization algorithm (Ott 1977).

Results

To examine a possible association of genetic substitutions in the two candidate loci—one including *SLC22A4* and *SLC22A5* and the other at *DLG5*—with susceptibility to CD in the Japanese population, we first examined DNA sequences from 48 CD patients in 438–976 bp genomic regions, including the five major genetic

Table 1 List of primers in this study

	Position	Primer		Product size
		Forward	Reverse	
Amplification for mutation analysis and genotyping				
<i>SLC22A4</i>	Ex9	AACTCTGGTAGGCAAAGAAGCTC	GTCCTACTTACCATTTCACCTTC	438
<i>SLC22A5</i>	Promoter	CTAGGATCGTTAATCGTGAAG	CTGAGCAGGAAGAAGATGAG	866
<i>DLG5</i>	Ex3	TCACTTTCAGTTCTACCTGCTAC	TCTAGGAGACAGTGGTAGGG	641
	Ex23	GAGACAGGATGCTCACAGCTTC	AACTCCTGAAGACCTGGTGTG	527
	Ex26	CTGATCGTGTTCCTTCTGTGCTG	AGGTCTCAAGGCTACATCTCCTC	976
Sequencing for mutation analysis and genotyping				
<i>SLC22A4</i>	Ex9	CATGCACAATGTCATCTGCC	ATAGGAGGACTCTCTGGGCAC	
<i>SLC22A5</i>	Promoter	GGACTCGGACCCCAAGGCCTC	AAGAAGATGAGGCGCTGGAAG	
<i>DLG5</i>	Ex3	ACTTTCAGTTCTACCTGCTACCG		
	Ex23	ATGCTCACAGCTTCCTGAGGTC	AAGACCTGGTGTGCGGCCTG	
	Ex26	CCTTCTGTGCTGTGGTCCAG	CGTTATGCCTTCTGACCCATC	