Table 3. Candidate Genes Within 500-kb Perimeter of D11S902 and D18S464

Marker	Position (kbp)	Symbol	Description
	16765.8-16992.5	PLEKHA7	Pleckstrin homology domain containing, family A member 7
	16950.0-16983.2	LOC644889	Similar to large subunit ribosomal protein L36a
	17030.4-17031.0	OR7E14P	Olfactory receptor, family 7, subfamily E, member 14 pseudogene
	17052.5-17022.8	PRS13	Ribosomal protein S13
	17052.8-17052.9	RNU14	RNA, U14 small nucleolar
	17053.9-17054.0	RNU14B	RNA, U14B small nucleolar
	17067.9-17147.9	PIK3C2A	Phosphoinositide-3-kinase, class 2, alpha polypeptide
	17205.8-17214.8	LOC91561	Similar to ribosomal protein S2; 40S ribosomal protein S2
	17254.9-17309.6	NUCB2	Nucleobindin 2
	17330.0-17355.4	DKFZp686024166	Hypothetical protein DKFZp686024166
	17363.4-17366.8	KCNJ11	Potassium inwardly rectifying channel, subfamily J, member 11
	17371.0-17455.0	ABCC8	ATP-binding cassette, subfamily C (CFTR/MRP), member 8
D11S902	17403		
	17472.0-17522.5	USH1C	Usher syndrome 1C (autosomal recessive, severe)
	17525.5-17624.1	OTOG	Otogelin
	17697.7-17700.3	MYOD1	Myogenic differentiation 1
	17714.1-17750.8	KCNC1	Potassium voltage-gated channel, Shaw-related subfamily, member 1
	17766.1-17991.2	DELGEF	Deafness locus-associated putative guanine nucleotide exchange factor
	9324.0-9325.4	LOC645604	Hypothetical protein LOC645604
	9324.9-9392.4	TWSG1	Twisted gastrulation homolog 1 (*Drosophila*)
	9465.5-9528.1	RALBP1	ralA-binding protein 1
	9523.1-9604.6	PPP4R1	Protein phosphatase 4, regulatory subunit 1
	9668.2-9668.6	LOC124242	Similar to keratin, type I cytoskeletal 18 (cytokeratin 18; K18, CK 18)
	9698.3-9852.5	RAB21	RAB31, member RAS oncogene family
D18S464	9819		
	9875.8-9878.2	TXNDC2	Thioredoxin domain containing 2 (spermatozoa)
	9904.0-9949.6	VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33 kDa

genes within 500-kb perimeters of D11S902 and D18S464, as well as to investigate the areas other markers (data not shown).

Stratifying the patients according to whether they had HLA-DR4 allowed us to examine potential relationships between susceptibility or resistance loci with HLA. No specific markers were detected in HLA-DR4-negative patients, though D11S902 was found to be weakly associated with DR4-positive patients (Table 4).

Next, the patients were classified into 2 groups, the severe group or the mild to moderate group, according to disease severity (Table 5).We defined severe disease as a total bilirubin of more than 5 mg/dL and/or a prothrombin time (PT) of less than 40% and mild to moderate

Table 4. Comparison of Allele Frequencies in DR4-Positive and DR4-Negative Patients

Susceptible Marker	DR4(+) (n = 61), %	DR4(−) (n = 20), %	*P*	Resistance Marker	DR4(+) (n = 61), %	DR4(−) (n = 20), %	*P*
D2S367	11.5	10.0	0.855	D1S252	3.3	0.0	0.412
D6S309	29.5	25.0	0.698	D1S2785	14.8	5.0	0.250
D9S273	24.6	5.0	0.056	D1S2800	3.3	0.0	0.412
D11S1320	92.7	95.0	0.724	D3S1580	9.8	5.0	0.481
D11S902	34.4	10.0	0.036	D4S405	26.2	30.0	0.742
D16S423	49.2	65.0	0.214	D4S2964	11.5	5.0	0.400
D17S938	23.0	25.0	0.851	D5S641	19.7	40.0	0.067
D18S464	26.2	25.0	0.913	D5S400	19.7	10.0	0.321
D18S68	9.8	10.0	0.983	D7S515	41.0	36.3	0.635
				D7S530	1.6	10.0	0.086
				D8S1771	1.6	5.0	0.401
				D10S249	22.0	25.0	0.497
				D10S1653	6.6	5.0	0.802
				D14S276	0.0	0.0	
				D15S1002	6.6	10.0	0.610
				D15S165	90.2	90.0	0.983
				D17S849	26.2	25.0	0.913

Table 5. Clinical Characteristics of Patients with Mild to Moderate and Severe AIH

Features	Mild to Moderate (n = 34)	Severe (n = 33)
Mean age (years)	55.7 (23-85)	56.1 (34-79)
Women:men	33:1*	22:11*
DR4(+):DR4(−)	25:9	22:11
ALT (nl: 7-45 U/L)	429.4 (47-1800)†	925.6 (128-2159)†
Bilirubin (nl: 0.3-1.2 mg/dL)	1.49 (0.4-4.5)†	11.86 (0.3-30.4)†
ALP (nl: 124-367 U/L)	456.3 (169-1180)	339.4 (131-1405)
IgG (nl: 800-2000 mg/dL)	3275.5 (1639-7248)	3114.2 (1134-5665)

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; nl, normal range.
*$P = 0.001$; †$P < 0.0001$.

disease as a total bilirubin of 5 mg/dL or less and a PT of 40% or more. All patients with severe disease had had acute exaggerating-phase episodes, in which total bilirubin had transiently risen to more than 5.0 mg/DL and/or PT had fallen to less than 40%, that improved after treatment. All patients with mild to moderate disease had not had any episodes of jaundice or symptoms of hepatitis. We excluded 14 patients because information on PT was lacking. From this, we observed that patients with severe disease were more likely to be male ($P = 0.001$) and that ALT ($P < 0.0001$) and total bilirubin ($P < 0.0001$) were significantly higher in patients with severe disease than in patients with mild to moderate disease (Table 5). No differences in the frequency of having susceptibility or resistance markers were seen over the clinical courses of the patients (Table 6).

Discussion

Although this is the first case-control association study to search for candidate genes of AIH pathogenesis throughout the whole genome, there have been several previous genomewide studies—of rheumatoid arthritis (RA),[22,23] autoimmune thyroid disease,[24] multiple sclerosis,[25] and systemic lupus erythematosus (SLE)[26,27] and of common diseases such as hypertension[28] and type 1 diabetes[29]—that attest to the effectiveness of microsatellite analysis. Most of these studies took multilocus, nonparametric approaches using affected-sibling pairs to scan novel disease susceptibility loci. In AIH, however, it is very rare for 2 or more family members to be affected—of the 130 AIH patients we have encountered in our hospital and in several regional hospitals in Nagano prefecture over the last 3 decades, only 2 patients had more than 2 affected members affected with AIH in their families. As such, there have been no reports of wide-scale searches for family clustering of AIH. In contrast, familial occurrence of primary biliary cirrhosis (PBC) is relatively high, up to 6.4%,[30] though the reason for the scarcity of multiplex families in AIH compared with other autoimmune diseases such as PBC, RA, and SLE is still unclear. We therefore performed a case-control association study, which did not require a large number of sib pairs for analysis. Such studies are performed by genotyping a set of anonymous markers in independent cohorts of affected and healthy individuals.

There are 2 common ways to investigate genetic associations of autoimmune diseases: the candidate gene approach, which is hypothesis driven by knowledge of an immunological process, and the genomewide association approach, in which the entire genome is searched at once in an unbiased fashion. The former approach is performed on gene polymorphisms of tumor necrosis factor α and cytotoxic T-lymphocyte antigen 4 in AIH patients.[15,16] However, the main bottleneck of the microsat-

Table 6. Comparison of Allele Frequencies Between Patients with Mild to Moderate and Patients with Severe AIH

Susceptible Marker	Mild-Moderate (n = 34), %	Severe (n = 33), %	P	Resistance Marker	Mild-Moderate (n = 34), %	Severe (n = 33), %	P
D2S367	8.8	12.1	0.659	D1S252	2.9	0.0	0.321
D6S309	23.5	33.3	0.373	D1S2785	8.8	18.1	0.261
D9S273	14.7	18.2	0.701	D1S2800	5.9	0.0	0.157
D11S1320	97.1	97.0	0.983	D3S1580	8.8	12.1	0.659
D11S902	29.4	30.3	0.937	D4S405	32.4	21.2	0.304
D16S423	50.0	54.5	0.710	D4S2964	5.9	9.0	0.617
D17S938	17.6	30.3	0.225	D5S641	38.2	18.1	0.069
D18S464	26.5	30.3	0.728	D5S400	11.8	18.1	0.461
D18S68	2.9	15.2	0.080	D7S515	38.2	45.5	0.549
				D7S530	5.9	3.0	0.573
				D8S1771	0.0	6.1	0.145
				D10S249	20.6	21.2	0.950
				D10S1653	5.9	9.0	0.617
				D14S276	0.0	0.0	
				D15S1002	8.8	3.0	0.317
				D15S165	85.3	90.9	0.479
				D17S849	32.4	18.1	0.183

ellite approach is sufficient marker density. For instance, Tamiya et al. used 27,039 microsatellite markers to perform their whole-genome association study of RA, using pooled DNA samples to reduce the costs and labor.[23] As we used only 400 microsatellite markers, each an average of 10.8 cM apart, we must concede there may be genes undetected by our study. This current genomewide scan in Japanese AIH patients found at least 9 candidate regions other than the HLA class II region to be associated with type 1 AIH susceptibility and up to 17 regions associated with resistance to AIH. We could not find any common loci among the susceptibility and resistance marker regions between our AIH patients and previously reported Japanese patients with RA or thyroid diseases (data not shown).[22-24] On chromosome 1, loci that specify ANA reactivity in SLE in humans and mice have been found.[27,31] ANA is increased in type 1 AIH, making this, and other likely susceptibility loci worth investigating. Although our current data did not find any significant linkages on chromosome 1, this region may be worth further analysis using narrow-interval microsatellite markers, as we did in the HLA region,[19] in order to test for any relationships.

Because the average length of linkage disequilibrium between disease-susceptible genes and nearby microsatellite alleles was more than 100 kb,[23] we therefore searched several candidate genes within a 500-kb perimeter of D11S902 and D18S464 (Table 3) and other markers (data not shown). However, none of the genes described here has been reported to be associated with autoimmune diseases. Nonetheless, several candidate genes found in this study are involved in interesting cell functions. For example, the protein encoded by phosphoinositide-3-kinase, class 2, alpha polypeptide (*PIK3C2A*) belongs to the phosphoinositide 3-kinase (PI3K) family. PI3 kinases play roles in signaling pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking. This protein contains a lipid kinase catalytic domain as well as a C-terminal C2 domain, a characteristic of class II PI3 kinases. C2 domains act as calcium-dependent phospholipid-binding motifs that mediate translocation of proteins to membranes and may also mediate protein–protein interactions.[32] The protein encoded by *KCNJ11* is an integral membrane protein and inward-rectifier-type potassium channel. In addition, *ABCC8* is a member of the superfamily of ATP-binding cassette transporters. Mutations in these genes are causes of familial persistent hyperinsulinemic hypoglycemia of infancy.[33] The *KCNJ11* E23K variant was reported to be associated with type 2 diabetes.[34] The protein encoded by receptor-interacting protein kinase; RIP1 (*RALBP1*) and NF-kappaB activation is associated with tumor necrosis factor receptor 1 signaling, which initiates several cellular responses including apoptosis.[35] Last, the protein encoded by *VAPA* is a type IV membrane protein. It is present in the plasma membrane and intracellular vesicles. This protein may function in vesicle trafficking, membrane fusion, protein complex assembly, and cell motility.[36] Recently, gene expression profiles of the liver tissues of AIH patients were shown using cDNA microarrays containing 1,080 cDNA clones.[37] Several genes were up-regulated in the liver tissues of AIH patients compared with those with PBC, chronic hepatitis C, and nonalcoholic steatohepatitis. However, no such genes were found in our current study, except for MHC class II DR.

Interestingly, D11S902 was found to be weakly associated with DR4-positive patients, whereas D18S464 was associated with both DR4+ and DR4−. This potentially important finding needs confirmation in studies with larger numbers of cases.

To find genes associated with mild to moderate or severe disease, we divided the patients into 2 groups and compared our findings. However, there were no specific regions associated with severity of AIH, so we cannot yet clarify the regions that regulate the different clinical course of AIH.

In conclusion, we were able to use genomewide microsatellite analysis as an effective strategy for identifying positive associations between microsatellite markers and AIH. Our current study was preliminary in nature because of the small number of test cases and controls used and the limited number of markers. Further genomewide studies are needed in a second cohort or in a larger test group with more markers, in addition to analysis of the single-nucleotide polymorphisms of the positive marker sites found in our study. Future studies are needed to analyze the relationship between gene polymorphisms and the expression and functions of these gene products, as well as research on any therapy-effectiveness- or disease-severity-related genes. These results may provide the specific tools necessary for therapeutic intervention of AIH.

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Lack of association between FCRL3 and FcγRII polymorphisms in Japanese type 1 autoimmune hepatitis

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Abstract Autoimmune hepatitis (AIH) is an organ-specific autoimmune disease characterized by chronic inflammation of the liver. Although the HLA-DRB1*0405 allele is associated with type 1 AIH in Japanese, the exact genetic etiology of AIH remains undefined. Recently, polymorphisms of Fcγ receptors (FcγR) and Fc receptor-like gene 3 (FCRL3) were linked to a variety of autoimmune diseases, and may be at least partially responsible for susceptibility to AIH. In this study, we genotyped FcγRIIA, FcγRIIB, and four FCRL3 polymorphisms in 87 Japanese patients with type 1 AIH and 97 ethnically matched controls using the TaqMan assay. Although we were able to detect significantly lower serum IgG concentrations in AIH patients specifically with the FCRL3−110A/A genotype, we observed no difference in the distribution of the genotypes between patients and controls, implying that susceptibility to type 1 AIH in Japanese patients is not influenced by FcγRIIA, FcγRIIB, or FCRL3 polymorphisms.

Introduction

Autoimmune hepatitis (AIH) is an organ-specific autoimmune disease characterized by chronic inflammation of the liver, elevated transaminase levels, hypergammaglobulinemia, serum autoantibodies, histologic evidence of interface hepatitis, and a favorable response to immunosuppressive treatment [1–3]. Type 1 AIH is distinguished by the presence of circulating antinuclear antibodies and/or smooth muscle antibodies, and is the major form of AIH in Japanese and Caucasoid adults. Although this disease is believed to result from a combination of genetic and environmental factors, its exact etiology remains unidentified. In previous studies, human leukocyte antigens (HLA) DRB1*0301 and/or DRB1*0401 alleles in Caucasians [4–6] and the DRB1*0405 allele in Japanese [7,8] were identified

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as independent determinants of AIH susceptibility. However, HLA alone does not explain the entire genetic predisposition to AIH, mainly since at least 30–40% of patients with the disease do not carry the most common susceptibility alleles. In this regard, non-HLA genes may also contribute to the disease process [9].

Recent research has identified a new family of genes, the Fc receptor-like genes, FCRLs [10] (also known as FcRHs [11,12], IRTAs [13,14], or SPAPs [15]), whose clusters locate near 1q21. FCRLs show high structural homology with classical Fcγ receptor genes, and these receptors may be at least partially responsible for susceptibility to autoimmune diseases, though their ligands and functions are not yet known. Kochi *et al.* [16] reported that a single nucleotide polymorphism (SNP) in the promoter region of FCRL3 is associated with susceptibility to rheumatoid arthritis, autoimmune thyroid disease, and systemic lupus erythematosus in the Japanese population. This polymorphism alters the binding affinity of nuclear factor-κB and regulates FCRL3 expression. Another study from Japan supports this genetic association of the FCRL3 promoter polymorphism with rheumatoid arthritis [17]. Additionally, we previously found that the FCRL3−110 allele is associated with susceptibility to Japanese autoimmune pancreatitis and is positively correlated with serum IgG4 concentrations, which is associated with disease activity [18].

There are three Fcγ receptor type II (FcγRII) genes (FcγRIIa, FcγRIIb, and FcγRIIc) that have been physically mapped around a 200 kb span at 1q23 [19]. FcγR receptors confer potent cellular effector functions to the specificity of IgG. FcγR-induced leukocyte functions, including antibody-dependent cellular cytoxicity, phagocytosis, cytokine production, and regulation of antibody production, are essential for host defense and immune regulation. FcγRIIa is the most widely distributed subclass, and is expressed on virtually all myeloid cells, including platelets. The expression of FcγRIIb is restricted to phagocytes and B cells. Although FcγRIIa and FcγRIIb polymorphisms in particular have been reported to be associated with genetic susceptibility to rheumatoid arthritis and systemic lupus erythematosus [19–22], these genes have not been examined with respect to AIH susceptibility. As such we hypothesized that FCRL3, FcγRIIa, and FcγRIIb polymorphisms might be associated with AIH in the Japanese population. To test this hypothesis, we typed four FCRL3 SNPs, FcγRIIa, and FcγRIIb SNPs in patients with AIH and controls.

Materials and methods

Subjects

A total of 87 patients with type 1 AIH (73 women, median age 56 years old, range 23–85 years) and 97 healthy subjects participated in this study. They were all residents of Nagano Prefecture, Japan, and their racial backgrounds were all Japanese. All patients had been diagnosed according to the scoring system from the International Autoimmune Hepatitis Group [23] and were classified as having type 1 AIH based on antibody profiles. The HLA DRB1*0405 allele was frequently found in 56 (64%) of the patients with type 1 AIH, as previously published [8]. All patients were negative for the hepatitis B surface antigen, antibody to hepatitis B core antigen, and antibody to hepatitis C or HCV RNA in serum. Study protocols were reviewed and approved by the appropriate institutional review boards, and written informed consent was obtained from all subjects.

DNA extraction and detection of FCRL3, FcγRIIa, and FcγRIIb polymorphisms

Genomic DNA from patients and healthy individuals were isolated by phenolic extraction of sodium dodecyl sulfate-lysed and proteinase K-treated cells, as described previously [24].

A total of six SNPs (FcγRIIa H/R131, FcγRIIb I/T232, and FCRL3−169, −110, +358, and +1381) were genotyped using the SNP Genotyping Kit (Applied Biosystems, Tokyo, Japan). Polymerase chain reaction was performed with a TaqMan Assay for Real-time PCR (7500 Real Time PCR System; Applied Biosystems), following the manufacturer's instructions.

HLA typing

HLA class I and II alleles were determined using the Micro SSPTM DNA Typing Kit (One Lamda, Canoga Park, CA). DNA typing of DRB1 and DQB1 alleles was performed by polymerase chain reaction-restriction fragment length polymorphism analysis, as previously described [24].

Statistical analysis

The significance of allele distribution in AIH patients and normal controls was tested by the χ^2 test for two-by-two or two-by-three comparisons, and a P value of ≤ 0.05 was considered significant. We also compared the genotypes of each polymorphism with clinical characteristics of the patients using the χ^2 test for two-by-two or two-by-three comparisons, as well as the Student's t-test. Hardy–Weinberg equilibrium and linkage disequilibrium were analyzed using

Table 1 FCRL3 polymorphisms in patients with type 1 AIH and healthy controls

Genotype	AIH (n=87)	Controls (n=97)	χ^2	P
−169				
C/C	13 (14.9)	11 (11.3)	0.26	0.61
C/T	45 (51.7)	51 (52.6)	0.00	0.97
T/T	29 (33.3)	35 (36.1)	0.05	0.81
−110				
A/A	4 (4.6)	2 (2.1)	0.30	0.58
A/G	34 (39.1)	35 (36.1)	0.07	0.79
G/G	49 (56.3)	60 (61.9)	0.38	0.54
+358				
C/C	13 (14.9)	11 (11.3)	0.26	0.61
C/G	45 (51.7)	51 (52.6)	0.00	0.97
G/G	29 (33.3)	35 (36.1)	0.05	0.81
+1381				
A/A	13 (14.9)	11 (11.3)	0.26	0.61
A/G	45 (51.7)	51 (52.6)	0.00	0.97
G/G	29 (33.3)	35 (36.1)	0.05	0.81

Table 2 FcγRIIa and FcγRIIb polymorphisms in patients with type 1 AIH and healthy controls

Genotype	AIH (n=87)	Controls (n=97)	χ^2	P
FcγRIIa/131				
R/R	4 (4.6)	5 (5.2)	0.03	0.87
R/H	30 (34.5)	32 (33.0)	0.00	0.95
H/H	53 (60.9)	60 (61.9)	0.00	0.98
FcγRIIb/232				
I/I	3 (3.4)	4 (4.1)	0.02	0.88
I/T	36 (41.4)	28 (28.9)	2.64	0.10
T/T	48 (55.2)	65 (67.0)	2.24	0.14

Gene Pop on the Web (http://wbiomed.curtin.edu.au/genepop/).

Results

Polymorphisms in the FCRL3, FcγRIIa, and FcγRIIb genes were analyzed in our 87 AIH patients and 97 healthy subjects and summarized in Tables 1 and 2. The observed genotype frequencies for patients and controls were all in Hardy–Weinberg equilibrium. The allelic frequencies in controls have been reported previously [18], and were similar to those reported in other Japanese populations prior to this study [16,20]. Analysis of allelic frequencies revealed no significant difference between AIH patients and control subjects for FCRL3, FcγRIIa, or FcγRIIb polymorphisms.

As observed in a study by Kochi *et al.*[16], FCRL3 polymorphisms are all linked (data not shown), so we therefore inferred three common haplotypes (Table 3). The haplotype frequency in controls was similar to previously reported Japanese control subjects [16], and there was no significant difference found between AIH patients and controls.

Since we previously reported that the HLA DRB1*0405 allele is associated with type 1 AIH in Japan [7,8], we further investigated the genetic association between this allele and the FCRL3, FcγRIIa, and FcγRIIb polymorphisms (Table 4). Analysis of allelic frequencies revealed no significant difference between patients with and without the HLA DRB1*0405 allele and these polymorphisms in χ^2 tests for two-by-three comparisons.

We also found no significant difference between FCRL3, FcγRIIa, and FcγRIIb polymorphisms and clinical characteristics in relation to age, sex, or serum levels of ALT. As shown in Fig. 1, mean serum IgG concentrations were significantly lower in patients with genotype FCRL3−110 A/A than in those with -110G/G (2144.0±391.1 mg/dL vs. 3307.4±180.3 mg/dL; P=0.042 by Student's t-test).

Table 4 Association between HLA DRB1*0405 and FCRL3, FcγRIIa, and FcγRIIb polymorphisms in patients with type 1 AIH

	DRB1*0405 (+) (n=56) n (%)	DRB1*0405 (−) (n=31) n (%)	P
FCRL3/−169			
C/C	10 (17.9)	3 (9.7)	0.59
C/T	28 (50.0)	17 (54.8)	
T/T	18 (32.1)	11 (35.5)	
FCRL3/−110			
A/A	2 (3.6)	2 (6.5)	0.56
A/G	24 (42.9)	10 (32.3)	
G/G	30 (53.6)	19 (61.3)	
FcγRIIa/131			
R/R	2 (3.6)	2 (6.5)	0.65
R/H	21 (37.5)	9 (29.0)	
H/H	33 (58.9)	20 (64.5)	
FcγRIIb/232			
I/I	2 (3.6)	1 (3.2)	0.42
T/I	26 (46.4)	10 (32.3)	
T/T	28 (50.0)	20 (64.5)	

Discussion

In our previous studies, the HLA DRB1*0405 allele was determined to correlate with an increased prevalence of AIH in the Japanese population [7,8]. However, because none of the identified genetic markers were sufficient to fully explain disease etiology, a number of genes outside the major histocompatibility complex region were suspected to play a role in AIH susceptibility. For instance, polymorphisms of the CTLA-4, TNF-α, VDR, and Fas genes have all been identified as correlating with an increased prevalence of AIH, though these findings have been disputed [8,25–30]. Still, FCRL3 and FcγR genes emerged as attractive candidates for this study, since they have previously been implicated in other autoimmune diseases.

This study produced three main observations that warrant further comment. First, none of the FCRL3, FcγRIIa, or FcγRIIb alleles were found to be associated with susceptibility or resistance to type 1 AIH in our test group. Second, no genetic association between the HLA DRB1*0405 allele and FCRL3, FcγRIIa, or FcγRIIb alleles was found in patients

Table 3 Association of FCRL3 haplotypes in type 1 AIH patients and healthy controls

Haplotype					Frequency			
	−169	−110	+358	+358	Autoimmune hepatitis (n=174)	Healthy subjects (n=194)	χ^2	P
1	T	G	G	G	0.59	0.62	0.27	0.61
2	C	A	C	A	0.24	0.20	0.65	0.42
3	C	G	C	A	0.17	0.18	0.00	0.94

Values for n indicate two times the number of individuals since each person carries two haplotypes.

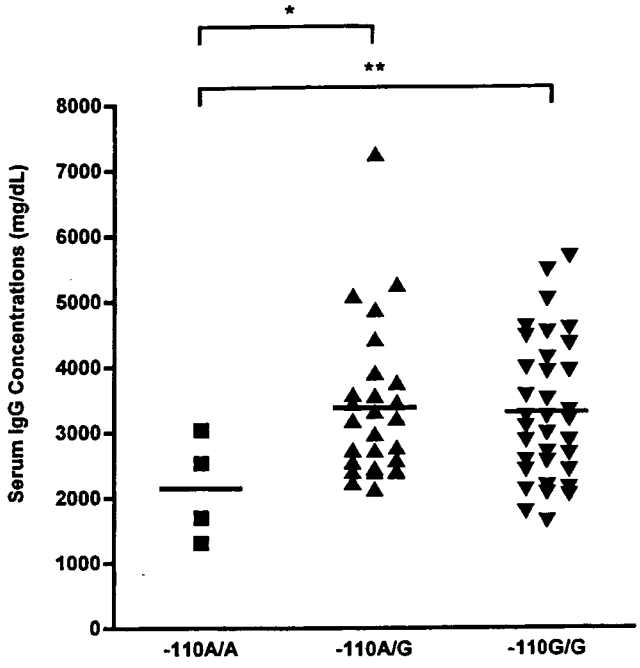

Figure 1 FCRL3−110 genotype and serum IgG concentrations in patients with type 1 AIH. Serum IgG levels were measured in 4 patients with −110A/A, 27 patients with −110A/G, and 33 patients with −110G/G. Solid lines indicate the mean values. *P=0.052, **P=0.042.

with AIH. Finally, mean serum IgG concentrations were significantly lower in patients with genotype FCRL3−110 A/A than in those with −110G/G, which suggests that the FCRL3 −110 allele might influence serum IgG concentrations in type 1 AIH.

FcγR receptors serve as a link between the humoral and cellular branches of the immune system [19]. FcγRIIa displays a G to A point mutation in the region specifying its ligand binding domain, causing an arginine to histidine amino acid substitution at position 131. A single T to C nucleotide change specifying an isoleucine or threonine at position 232 was recently described in the transmembrane region of FcγRIIb. FcγR polymorphisms influence the efficacy of cellular responses, and have been associated with inflammatory diseases and disease severity. A meta-analysis has confirmed the association between FcγRIIa-R131 and SLE [31].

The FCRL3 gene was recently discovered to be transcribed by nuclear factor-κB. Of particular note, the susceptibility C allele of the FCRL3−169 SNP has been shown to alter FCRL3 expression through nuclear factor-κB promoter binding, leading to higher FCRL3 expression on B cells [16]. FCRL3 molecules contain both immunotyrosine activation and inhibitory motifs, enabling them to both activate and inhibit signaling pathways, and are believed to be involved in regulating cellular signaling thresholds. FCRL3 polymorphisms have been shown to be associated with various autoimmune diseases such as rheumatoid arthritis, autoimmune thyroid disease and systemic lupus erythematosus in Japanese populations [16,17]. Furthermore, we previously reported that the FCRL3−110A/A allele is associated with autoimmune pancreatitis [18]. However, there were no correlations between FCRL3, FcγRIIa, and FcγRIIb polymorphisms and type 1 AIH in this study. These results suggest that AIH has a different immunogenic background than the above diseases with regard to these genes.

As reported by Hiraide et al.[25], Fas gene polymorphisms and haplotypes are associated with AIH in Japanese, and are predominantly present in DR4-positive AIH patients. Due to the fact that the HLA DRB1*0405 allele is associated with type 1 AIH in Japanese, we also sought to determine the correlation between HLA and polymorphisms of FcγR receptors and FCRL3. However, no association between the HLA DRB1*0405 allele and these polymorphisms was found in this study. We previously showed that two markers of the HLA DRB1*0405-DQB1*0401 haplotype and FCRL3−110 alleles were susceptible to autoimmune pancreatitis, though no association between this haplotype and FCRL3−110 alleles was found as well [18]. Since FcγR and FCRL3 are located on different chromosomes than HLA-DR, there is no linkage disequilibrium between these genes.

A previous study reported that the FCRL3−110 allele influences serum IgG4 concentrations in autoimmune pancreatitis, which is associated with disease activity [18]. In our study, serum IgG levels were significantly lower in AIH patients homozygous for the FCRL3−110A/A polymorphism than in those that were homozygous for the FCRL3−110G/G genotype. However, as there were only 4 patients with the −110A/A genotype in our cohort, it might be better to conclude this association following more studies with larger −110A/A test groups. Serum IgG4 levels were not detected in the present study since serum was not available in storage and there had been no prior association between disease activity and serum IgG4 concentration in AIH. Nonetheless, our results suggest that the FCRL3−110 allele may play a pivotal role in the secretion of IgG or IgG4 in patients with AIH and autoimmune pancreatitis, respectively. At present, we can only describe the observation of these correlations, but cannot yet provide a sound scientific basis for their occurrence. Further study is needed to clarify this interesting association.

In conclusion, we found that FcγRIIa, FcγRIIb, and FCRL3 polymorphisms are not associated with susceptibility to type 1 AIH in Japan. However, the FCRL3−110 polymorphism may be implicated in the secretion of IgG in AIH patients. Genetic variations associated with AIH susceptibility remain for further investigation. A genome-wide genetic association study of AIH has been conducted in our group.

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Efficacy and safety of 6-month iron reduction therapy in patients with hepatitis C virus-related cirrhosis: a pilot study

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Editorial on page 96

Background. Iron reduction therapy (IRT) has been recognized as beneficial for chronic hepatitis C patients. However, its efficacy for hepatitis C virus-related liver cirrhosis (LC-C) has not been elucidated. We evaluated the efficacy and safety of IRT for LC-C patients. ***Methods.*** Twenty-two LC-C patients were treated with biweekly phlebotomy and low iron diet for 6 months, in addition to regular hepatoprotective therapy. Nineteen sex- and age-matched patients who refused to receive IRT were used as controls. The efficacy of IRT was evaluated on the basis of biochemical parameters. ***Results.*** Of 22 patients receiving IRT, 19 completed the 6-month treatment. IRT significantly reduced serum levels of aspartate aminotransferase (from 89 to 57 U/L; $P = 0.003$), alanine aminotransferase (from 101 to 54 U/L; $P < 0.001$), and α-fetoprotein (from 28 to 12 ng/mL; $P = 0.003$). These changes were not observed in the controls. Two patients whose serum albumin concentrations were less than 3.6 g/dL at the beginning of IRT withdrew from IRT because of the new appearance of ascites. ***Conclusions.*** IRT improved the serum levels of aminotransferases and α-fetoprotein in LC-C patients and was generally safe; however, IRT should be performed in patients who maintain serum albumin concentrations of more than 3.6 g/dL.

Key words: iron reduction therapy, HCV-related cirrhosis, α-fetoprotein, albumin, protein-energy malnutrition

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Introduction

Chronic hepatitis caused by persistent hepatitis C vir (HCV) infection may lead to the development of liv cirrhosis (LC) and eventually to the complication hepatocellular carcinoma (HCC).[1] LC has been reco nized as one of the major risk factors of HCC and h patic failure in patients with persistent HCV infectio To prevent the development of HCC and the progre sion to hepatic failure in HCV-related LC (LC-C), it important to keep serum aminotransferase levels as lo as possible.[2]

Interferon therapy has diverse beneficial effects f HCV-related chronic liver diseases: promoting HC elimination, suppressing HCV replication, amelioratir the activity of hepatitis, and eventually preventing th progression of hepatic fibrosis and the occurrence HCC. However, its use is sometimes limited in elder patients and in patients with marked thrombocyt penia, diabetes, severe systemic arteriosclerosis, psychological disease, because of the increased risk harmful or irreversible adverse effects (e.g., cerebr hemorrhage, depression). Therefore, the establishmer of alternative strategies is required for such LC- patients.

Iron reduction therapy (IRT) was first introduced b Hayashi et al.,[3] based on the histochemical detection iron deposits in the liver, and then on the detection lysosomal iron stores in hepatocytes. Hepatic iron a cumulation induces enhanced generation of reactiv oxygen species (ROS), thus damaging hepatocytes.[4] Recently, a randomized controlled study of Japanes chronic hepatitis C patients showed that biweekl phlebotomy for 3 months significantly reduced serur alanine aminotransferase (ALT) levels.[6] It has been re ported that in HCV-infected patients, intrahepatic iro accumulation and the resultant ROS production er hances as hepatic fibrosis progresses,[7] suggesting tha IRT might be useful for LC-C patients. However, fc

patients with compensated LC-C, the efficacy and safety of IRT, that is, the combination of phlebotomy and a low-iron diet, has not been previously elucidated. Therefore, we planned this pilot study to evaluate it.

Materials and methods

Patients

From October 2003 to March 2005, 22 LC-C patients (10 men and 12 women, 66 ± 11 years old) entered this pilot study after informed consent was obtained. All patients were positive for serum HCV-RNA and had abnormal serum ALT levels. Serum aspartate aminotransferase (AST) and ALT levels of less than 35 U/L were defined as the normal ranges. Basically, the diagnosis of LC was made according to the histological findings of percutaneous liver biopsy, and was confirmed by imaging findings (e.g., liver surface irregularity, swelling of the left and caudal lobes, presence of splenomegaly, and development of esophageal or gastric varices) and by laboratory data such as platelet counts of less than $10.0 \times 10^4/\mu L$[8] and serum hyaluronic acid levels of more than 237 ng/mL.[9] All 22 patients had splenomegaly, and seven of these had esophageal varices. Twenty of these patients had platelet counts of less than $10.0 \times 10^4/\mu L$, and 18 had serum hyaluronic acid levels of more than 237 ng/mL. Three of the 22 patients refused liver biopsy, so the diagnosis of LC was made using imaging findings and a formula for estimating liver cirrhosis proposed by Ikeda et al.[8] Its accuracy for diagnosing LC was as high as 91.2%. The exclusion criteria at entry were (1) previous interferon therapy within 6 months; (2) decompensated LC (Child-Pugh classification B or C); (3) hemoglobin values of less than 12 g/dL; (4) serum albumin concentrations of less than 3.3 g/dL; (5) severe complications such as cardiac, pulmonary, renal, or hematological disease; and (6) pregnancy. All patients underwent hepatoprotective therapy such as ursodeoxycholic acid or glycyrrhizin injection for more than 6 months before entry. Of the 22 patients, eight had been administered oral branched-chain amino acids for more than 6 months before entry. None had received regular administration of oral diuretics or albumin infusion. These therapies were not changed after this study was begun.

When informed consent was requested of the LC-C patients, some of them refused to participate in this study. Of these, 19 age- and sex-matched patients were selected and used as controls for the comparison of biochemical markers. These patients continued only hepatoprotective therapy for 6 months or more.

When IRT was begun, the body mass index (BMI) was calculated. Patients were considered to have hypertension if their systolic/diastolic pressure was greater than 140/90 mmHg, or if they were taking antihypertensive drugs. Patients were considered to have diabetes if they had a fasting glucose level equal to or higher than 126 mg/dL, or if they were taking insulin or oral hypoglycemic drugs.

Histological examination

Percutaneous ultrasonography-guided liver biopsy was performed before entry into the study, and the liver specimens were fixed in 10% formalin and stained using hematoxylin-eosin and the Azan-Mallory methods. The presence of hepatic steatosis was defined as macrovesicular fat accumulation in more than 30% of the hepatocytes affected.

IRT

IRT consisted of a periodic phlebotomy and a low-iron diet. Removal of 200 mL of blood from female patients with body weight of less than 60 kg or of 400 mL from other patients was repeated biweekly. Phlebotomy was planned to be continued for 6 months, but was discontinued according to the following criteria: (1) hemoglobin values of less than 10 g/dL, (2) serum ferritin levels of less than 10 ng/mL, (3) the appearance of adverse effects, or (4) patient's refusal to continue phlebotomy. In addition, all patients in the IRT group were advised to reduce their iron-rich food intake during the treatment and were instructed by a registered dietitian. To aid with compliance, each patient was given a comprehensive list of iron-rich foods that they were to avoid, as well as instructions on how to complete dietary records, which required a listing of all food and drink consumed over a 3-day period once every 3 months throughout the treatment. All patients in the IRT group were instructed to reduce their consumption of beans, shellfish, green vegetables, meat, and seaweed and replace them with refined carbohydrates. Dietary energy (30 kcal/kg of body weight/day), nutritional balance, and iron intake (6 mg/day) during the IRT were assessed based on the dietary records by using the nutrition analysis software BASIC-4 for Windows version 2.0 (Kagawa Nutrition University Publishing Division, Tokyo, Japan).

Laboratory tests

Complete blood counts, including hemoglobin values and platelet counts, and biochemical parameters such as serum AST, ALT, albumin, bilirubin, α-fetoprotein (AFP), hyaluronic acid, and ferritin levels were measured monthly using standard automated analyzers. The amounts of serum HCV-RNA were measured by the

Amplicor monitoring method (Roche Diagnostic System, Basel, Switzerland). In the IRT group, blood was drawn for tests just before phlebotomy was performed.

Ethics

This study was carried out in accordance with the World Medical Association Helsinki Declaration, and was approved by the ethics committee of Showa Inan General Hospital. All patients gave their informed consent to participate in the study.

Statistics

Statistical analyses were performed using SPSS software 11.0J for Windows (SPSS, Chicago, Illinois, USA). Qualitative variables were expressed as percentages and compared using the χ-squared test. Quantitative data were expressed as means and standard deviations and were compared using the unpaired or paired two-tailed t test. A probability value of less than 0.05 was considered to be statistically significant.

Results

Effects of IRT on laboratory tests

Of the 22 patients receiving IRT, 19 completed the 6-month treatment. There were no differences in the patients' backgrounds between the IRT and the control groups (Table 1). Serum AST and ALT levels in the IRT group were significantly decreased after the treatment (from 89 ± 33 to 57 ± 19 U/L; $P = 0.003$, and from 101 ± 44 to 54 ± 24 U/L; $P < 0.001$, respectively) (Figs. 1 and 2), but remained unchanged in the control group (from 84 ± 26 to 82 ± 23 U/L, and from 93 ± 26 to 91 ± 27 U/L, respectively) (Fig. 2). Serum AFP levels in the IRT group were also significantly decreased after the treatment (from 28 ± 20 to 12 ± 6 ng/mL; $P = 0.003$), but were constant in the control group (from 32 ± 24 to 31 ± 24 ng/mL) (Fig. 2). As a result of the IRT, the hemoglobin values and serum ferritin levels were decreased (from 14.5 ± 1.1 to 11.7 ± 1.9 g/dL; $P < 0.001$, and from 237 ± 145 to 26 ± 21 ng/mL; $P < 0.001$, respectively) (Fig. 2). The platelet counts were significantly increased after the IRT (from 9.2 ± 2.9 to 11.7 ± 2.5 × 10^4/μL; $P = 0.003$) (Fig. 2). Serum albumin, bilirubin, and hyaluronic acid levels remained unchanged after the treatment (Fig. 2).

Clinical parameters after 6 months of IRT

Several clinical parameters after 6 months of IRT were compared with those of the controls. Serum AST and ALT levels were significantly lower in the IRT group than in the control group (57 ± 19 vs. 82 ± 23 U/L; $P = 0.001$, and 54 ± 24 vs. 91 ± 27 U/L; $P = 0.003$, respectively) (Fig. 2). Serum AFP levels were also lower in the IRT group than in the control group after 6 months (12 ± 6 vs. 31 ± 24 ng/mL; $P = 0.042$) (Fig. 2). The hemoglobin values and serum ferritin levels significantly dropped in the IRT group compared with those in the control group (11.7 ± 1.9 vs. 13.8 ± 1.1 g/dL; $P < 0.001$, and 26 ±

Table 1. Baseline characteristics of study groups

	IRT ($n = 19$)	Control ($n = 19$)	P
Age (years)	67 ± 9	65 ± 6	0.559
Sex (male:female)	8:11	8:11	1.000
BMI (kg/m^2)	23.3 ± 2.5	22.0 ± 2.5	0.343
Hypertension (n)	10	6	0.325
Diabetes (n)	4	2	0.660
Steatosis (n)	3	2	1.000
Hemoglobin (g/dL)	14.5 ± 1.1	14.0 ± 1.2	0.375
Platelet (×10^4/μL)	9.2 ± 2.9	8.5 ± 3.6	0.517
Albumin (g/dL)	4.0 ± 0.4	3.9 ± 0.3	0.378
Bilirubin (mg/dL)	0.8 ± 0.3	1.1 ± 0.4	0.519
AST (U/L)	89 ± 33	84 ± 26	0.609
ALT (U/L)	101 ± 44	93 ± 26	0.540
Iron (μg/dL)	161 ± 53	160 ± 45	0.935
Transferrin saturation (%)	45.0 ± 14.6	48.4 ± 15.6	0.583
Ferritin (ng/mL)	237 ± 145	222 ± 104	0.410
AFP (ng/mL)	28 ± 20	32 ± 24	0.642
Hyaluronic acid (ng/mL)	292 ± 203	286 ± 110	0.438
HCV-RNA (kIU/mL)	639 ± 416	672 ± 412	0.859

Quantitative data were expressed as means ± SD. P values were calculated using the unpaired two-tailed t test or the χ-squared test

IRT, iron reduction therapy; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AFP, α-fetoprotein, HCV-RNA; circulating hepatitis C virus RNA

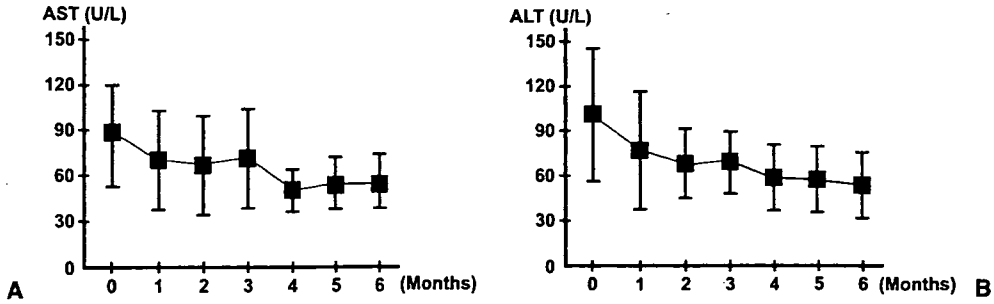

Fig. 1A,B. Changes in serum aminotransferase levels during iron reduction therapy (IRT). Serum aspartate aminotransferase (*AST*) (**A**) and alanine aminotransferase (*ALT*) levels (**B**) were significantly decreased by IRT. Data were expressed as means ± SD

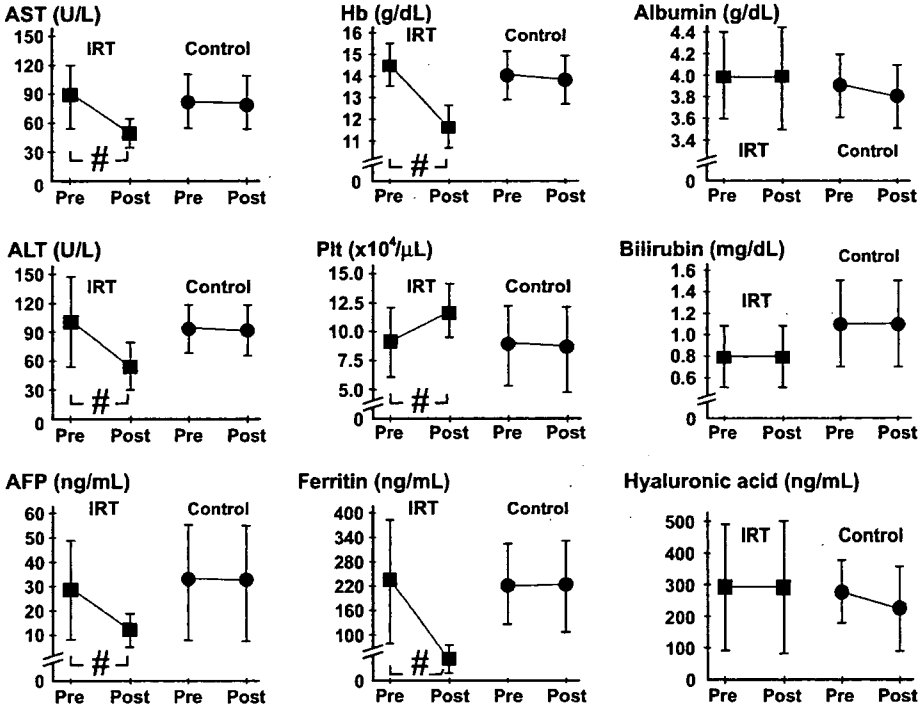

Fig. 2. Comparison of changes in the representative biochemical markers. Data were expressed as means ± SD. #, $P < 0.05$ compared with the pretreatment (paired two-tailed t test). *Hb*, hemoglobin; *Plt*, platelets; *AFP*, α-fetoprotein

21 vs. 223 ± 111 ng/mL; $P < 0.001$, respectively) (Fig. 2). The platelet counts were higher in the IRT group than in the controls (11.7 ± 2.5 vs. 8.4 ± 3.7 × 10^4/μL; $P = 0.004$) (Fig. 2). On the other hand, serum albumin, bilirubin, and hyaluronic acid levels did not differ between the two groups after 6 months (Fig. 2).

Association between improvement of clinical parameters and iron reduction

To examine whether iron reduction contributed to the improvement of serum aminotransferase levels in this study, the patients receiving IRT were divided into two groups according to their serum ferritin levels as measured after 6 months of treatment (more or less than 10 ng/mL), and the changes in their clinical parameters were compared. As shown in Table 2, six patients reached serum ferritin levels of less than 10 ng/mL after 6 months of IRT. Serum AST, ALT, and AFP levels after the treatment were similar in the two groups, but the change in AST levels was significantly greater in patients with serum ferritin levels of less than 10 ng/mL than in patients with serum ferritin levels of more than 10 ng/mL (Table 2). The changes in ALT and AFP levels tended to be greater in patients with serum ferritin levels of less than 10 ng/mL (Table 2).

Adverse effects

IRT was generally tolerated in LC-C patients. However, three of 22 LC-C patients withdrew from IRT. One patient refused phlebotomy because of the pain experienced at the venous puncture. The remaining two patients showed a new appearance of ascites due to decreased serum albumin levels. One of these patients was a 41-year-old obese man suffering from diabetes, and the other was a 70-year-old woman with hypertension. The serum albumin levels of these patients at the beginning of IRT were 3.5 and 3.4 g/dL, respectively. Discontinuance of phlebotomy and addi-

Table 2. Comparison between IRT group patients with serum ferritin concentrations more and less than 10 ng/mL after 6 months of treatment

	Ferritin < 10 (n = 6)	Ferritin > 10 (n = 13)	P
After 6 months of treatment			
Hemoglobin (g/dL)	10.2 ± 1.1	12.3 ± 1.9	**0.016**
Platelet (×10⁴/μL)	12.7 ± 2.1	11.1 ± 2.7	0.244
Albumin (g/dL)	4.1 ± 0.8	3.9 ± 0.3	0.682
AST (U/L)	51 ± 13	59 ± 22	0.379
ALT (U/L)	55 ± 27	53 ± 24	0.882
Ferritin (ng/mL)	6 ± 2	35 ± 20	**<0.001**
AFP (ng/mL)	9 ± 6	13 ± 6	0.244
Changes (6 month/0 month)			
AST (%)	53 ± 15	78 ± 30	**0.045**
ALT (%)	43 ± 13	65 ± 28	0.056
AFP (%)	40 ± 14	58 ± 24	0.144

Quantitative data were expressed as means ± SD. P values were calculated using the unpaired two-tailed t test. AST, aspartate aminotransferase; ALT, alanine aminotransferase; AFP, α-fetoprotein

tional oral administration of spironolactone resulted in the rapid disappearance of the ascites. Albumin infusion was not performed. In the control group, neither the new appearance of ascites nor HCC was observed.

Discussion

This pilot study demonstrated that IRT significantly improved the serum levels of aminotransferases and AFP for patients with LC-C. Moreover, IRT can be performed safely in patients with compensated LC-C. As far as we know, this is the first study concerning the efficacy and safety of IRT for LC-C patients.

The remarkable characteristic of this study is that only compensated LC patients were allocated. Histological diagnosis of LC only by ultrasonography-guided liver biopsy sometimes leads to misinterpretation because of a high sampling error.[8,10] In this study, the diagnosis of LC was made fundamentally according to the histological findings, and was confirmed by imaging and laboratory findings. Thus, the diagnosis of LC is considered to be valid in this study.

Six months of IRT significantly decreased serum aminotransferase levels, which may have been caused by reduced iron-induced hepatotoxicity. As shown in Table 2, the efficacy of iron reduction is supported by the fact that the degree of improvement of serum AST levels was greater in patients with serum ferritin levels of less than 10 ng/mL than in those who showed with levels of more than 10 ng/mL after the treatment. The probability that the improvement in serum aminotransferase levels resulted from other hepatoprotective therapies such as ursodeoxycholic acid or glycyrrhizin injection is considered to be very low, because the dose of these hepatoprotective agents was unchanged during this study and because these agents were also administered to the control group, in which the serum aminotransferase levels did not change.

Another notable finding in this study is that IRT significantly reduced serum AFP levels. Until now, only low-dose long-term interferon injection therapy has been recognized to improve serum AFP levels.[11] Since a high level of AFP is suggested to be one of the major risk factors for the development of HCC,[12,13] lowering the serum AFP levels is important to prevent HCV-related hepatocarcinogenesis. Hepatic iron accumulation induces mitochondrial abnormalities and enhanced ROS generation,[14] causing the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG)[14,15] and p53 mutation,[16] which may eventually lead to the initial step toward HCC. It has also been reported that hepatic iron accumulation disrupts the balance between hepatocyte proliferation and apoptosis in transgenic mice expressing HCV polyprotein[14] and in patients with chronic hepatitis C.[17] Furthermore, an in vitro study using mouse hepatocytes revealed that iron overload promoted *Cyclin D1* expression and accelerated hepatocyte division.[18] In fact, it has been demonstrated that IRT significantly lowered hepatic 8-OHdG levels and reduced the incidence rate of HCC in patients with chronic hepatitis C.[19] Therefore, maintaining hepatic iron contents as low as possible by long-term IRT might be useful for preventing HCV-related hepatocarcinogenesis, especially for patients with LC-C. Verifying this requires long-term follow-up observations.

There is a possibility that IRT, especially periodic phlebotomy, can decrease serum albumin concentrations. It has been unclear whether IRT is indicated for patients with compensated cirrhosis and serum albumin levels of less than 3.7 g/dL. This study demonstrated for the first time that, even in compensated cirrhosis, regu-

lar repeated phlebotomy is risky for patients with serum albumin levels of less than 3.6 g/dL. Therefore, in such patients, adjustment of phlebotomy (e.g., reduction of removed blood volume, extension of the interval between phlebotomies) is needed.

It is evident that a low-iron diet plays an important role in ameliorating the effect of IRT.[20] Many compensated LC-C patients have subclinical protein energy malnutrition (PEM);[21,22] excessive dietary iron restriction would worsen it. Careful nutritional evaluation and appropriate pharmacological interventions to improve PEM, such as regular branched-chain amino acid supplementation and a late-evening snack before starting IRT, and repeated education by dietitians, are essential if patients are to complete IRT safely and efficiently.

Long-term interferon therapy has been demonstrated to reduce development of HCC and improve survival of LC-C patients.[23] However, interferon therapy is not perfect as it has unpleasant adverse effects such as fever and appetite loss and is expensive. In addition, interferon therapy is risky for elderly patients and for patients with severe thrombocytopenia or depression. On the other hand, IRT has few adverse effects and is not so expensive; IRT can be performed safely in patients with cirrhosis with thrombocytopenia or psychological diseases. Thus, we think that IRT might compensate for the imperfections of interferon therapy in LC-C patients.

Concerning IRT for LC-C patients, there are some points to be evaluated hereafter. First, to confirm these beneficial effects of IRT, large-scale long-term prospective studies of compensated LC-C patients are needed. Second, since iron deposition enhances collagen synthesis in hepatic stellate cells and promotes the progression of hepatic fibrosis,[24] it would be of great value to evaluate whether long-term IRT can cause regression of hepatic fibrosis in patients with LC-C. Finally, the establishment of safe iron reduction strategies for LC-C patients having serum albumin levels of less than 3.6 g/dL, for example, the development of a new method of phlebotomy that removes only erythrocytes and transfuses the remainder into the vein, would be beneficial.

In conclusion, we demonstrated that IRT significantly reduced serum aminotransferases and AFP levels in LC-C patients. IRT was generally safe; however, IRT should be performed in patients with serum albumin concentrations of more than 3.6 g/dL.

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Genetic Analysis of Hepatitis A Virus Protein 2C in Sera from Patients with Fulminant and Self-limited Hepatitis A

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ABSTRACT

Background/Aims: To examine whether genetic differences in hepatitis A virus (HAV) are responsible for the range of clinical severities, we analyzed the HAV 2C genome, whose mutations have previously been shown to be important for enhanced replication in cell culture systems and to be related to virulence in simians.

Methodology: Serum samples from 45 Japanese patients with sporadic hepatitis A, comprising 9 patients with fulminant hepatitis (FH), 10 with severe acute hepatitis (AHs), and 26 with self-limited acute hepatitis (AH), were examined for HAV RNA.

Results: Compared with the sequence of wild-type HAV strain HM-175, the nucleotide sequences of 2C had homology of 89.0±0.6% in FH, 88.6±0.9% in AHs, and 89.0±1.6% in AH. Differences were not statistically significant among the three groups. Deduced amino acid sequences had homology of 97.6±0.4% in FH, 96.5±1.9% in AHs, and 96.8±1.7% in AH. The difference between FH and AH was statistically significant ($p<0.05$), although there were no specific nucleotide or amino acid substitutions.

Conclusions: Fulminant hepatitis patients had fewer amino acid substitutions in 2C, indicating the association between severity of hepatitis A and amino acid variations in 2C of HAV.

KEY WORDS: Hepatitis A; Fulminant hepatitis; Hepatitis A virus; 2C protein

ABBREVIATIONS: Hepatitis A Virus (HAV); Fulminant Hepatitis (FH); Acute Hepatitis (AH)

INTRODUCTION

Hepatitis A is still a major problem worldwide, not only in underdeveloped countries but also in industrialized nations. Because of improvements in sanitation, there have been no hepatitis A epidemics in Japan in recent years. However, sporadic cases of hepatitis A have not been rare of late. In fact, in the past several years, there has been an increase in the numbers of patients with sporadic hepatitis A, especially the more severe kind, visiting our hospital. Our analysis of the possible factors responsible for the severity of the disease in our patients revealed no significant differences in terms of background including age, suggesting that viral factors might be involved in determining the severity of the disease (1,2). Recently, Durst *et al.* reported a cluster of fulminant hepatitis A (3).

HAV is the sole member of the hepatovirus genus and a member of the picornavirus family. The single long open reading frame of the HAV genome encodes a large polyprotein that is cleaved by the viral protease to produce the P1, P2 and P3 regions. The P1 region encodes four structural proteins and the P2 and P3 regions encode seven nonstructural proteins. The 2C protein encoded by the P2 region of the polyprotein is highly conserved among picornaviruses (4).

Poliovirus has been used as a prototype of picornaviruses to understand the molecular biology of their replication. Poliovirus polypeptide 2C is a nonstructural protein involved in replication of the viral genome. HAV 2C gene mutations have previously been shown to be important for enhanced replication of the HM-175 strain in cell culture (5,6). Therefore, amino acid substitutions in the 2C protein might result in significant functional changes in the virus.

Despite advances in the understanding of HAV, a correlation between HAV genomes and the clinical status of hepatitis A has not been established. To examine the possibility of differences in hepatitis A viruses in terms of the different categories of hepatitis, we analyzed the viral genomes in sera from hepatitis A patients with a variety of clinicopathological features. We developed a technique for detecting HAV RNA with high frequency in the early convalescent phase of hepatitis A (7). We determined the full

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sequence of HAV from 3 cases of fulminant hepatitis and 3 cases of acute hepatitis (8), and we also reported that the nucleotide variations in the central part of the 5'NTR of HAV might be one of the factors that decide the severity of hepatitis A (2,9).

In the present study, we examined the clinicopathological features of hepatitis A and possible correlations with variations in the 2C protein of the HAV genome.

METHODOLOGY

Patients

Serum samples from 45 patients with hepatitis A in Japan were collected between 1986 and 1999 and stored at -20°C until analysis. Informed consent was obtained from patients or appropriate family members. These patients were diagnosed based on the positivity of IgM anti-HAV antibody (IgM-HA) in conjunction with compatible symptoms and laboratory findings.

The patients consisted of 9 with fulminant hepatitis (FH), 10 with severe acute hepatitis (AHs), and 26 with self-limited acute hepatitis (AH). Patients with a prothrombin time less than 40% of control were defined as AHs (10), and those with hepatic encephalopathy as FH. Patients with significant increases in serum blood urea nitrogen and creatinine (more than three times of the upper level of normal range) were judged to be undergoing acute renal failure (11). IgM anti-HBc antibody (IgM-HBc), HBsAg, and second generation anti-HCV antibody were examined in all cases. In the FH and AHs patients, HCV RNA, IgM anti-Epstein-Barr virus antibody (IgM-EBV), IgM anti-herpes simplex virus antibody (IgM-HSV), IgM anti-cytomegalovirus antibody (IgM-CMV), anti-smooth muscle antibody, liver kidney microsomal antibody-1 and anti-mitochondrial antibody were also examined. Histories of recent exposure to drugs and chemical agents as well as heavy alcohol consumption (>50g/d for >5 years) were also examined.

None of the patients had clinical or laboratory evidence of acquired immune deficiency syndrome.

Serological Markers

IgM-HA, IgM-HBc antibody and HBsAg were measured by commercial radioimmuno-assay kits (Abbott Laboratories, Chicago, IL), second generation HCV antibody was measured by enzyme immunoassay kit (Ortho Diagnostics, Tokyo, Japan), and HCV RNA was measured by nested RT-PCR as described by the authors (12). IgM-EBV, IgM-CMV, and IgM-HSV were examined by enzyme-linked immunosorbent assays. Anti-nuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody and anti-liver kidney microsomal-1 antibody were examined by fluorescent antibody method.

TABLE 1 Nucleotide Sequences of the Primers for RT-PCR and Sequencing

A primer for RT		
R-AL(7360-7328)	5'	GATTTAAGTCTGTATTCTATCATCTCTTTCTCC 3'
Primers for first PCR		
F3637(3637-3658)	5'	TGCTTGGATTGTCTGGAGTTCA 3'
R5259(5259-5238)	5'	TCACTTGCTTGGGCTTAGTTAC 3'
Primers for nested PCR and sequencing		
F3897(3897-3920)	5'	GGTTGTTCAGTTATTTCATGTGGC 3'
R5181(5181-5158)	5'	TATACACAAACCATCCTCCAACGA 3'

Primers for RT-PCR and for Direct Sequencing of the PCR Products

For amplification of the HAV sequence and for directly sequencing the amplified segment bidirectionally, we prepared 5 primers for RT-PCR and sequencing (**Table 1**). These primers were prepared based on the sequence reported by Cohen *et al.* (13).

Amplification of Serum HAV RNA for Direct Sequencing

RNA was extracted from sera using the acid guanidinium-phenol-chloroform method. Briefly, 50μL of samples was mixed with 1000μL of guanidinium solution (ISOGEN, Nippon Gene Co., Tokyo, Japan) and 200μL of chloroform, and precipitated with an equivalent volume of isopropanol. The RNA pellet was washed twice with 75% ethanol and dissolved in 11μL of RNase-free distilled water.

For reverse transcription, 11μL of the RNA was heat-denatured at 70°C for 10 min, and then chilled rapidly on ice. The RNA was mixed with 9μL of reaction mixture on ice. The final 20μL reaction mixture contained RNA from 50μL of serum, 2.5μM of antisense primer R-AL, 200 units of Superscript II reverse transcriptase (Superscript Preamplification System, GIBCO BRL, Gaitherburg, MD), 200μM of each dNTP, 50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM MgCl2, 0.001% gelatin, 5% DMSO and 10 units of RNase inhibitor (Takara, Kyoto, Japan). The reverse transcription reaction was done by incubation at 42°C for 90 min, followed by heating of the mixture at 70°C for 15 min.

The first round of PCR was performed in 50μL of reaction mixture containing 1/200 of cDNA, 1.0μM of outer antisense primer R5259 and sense primer F3637, 200μM of each dNTP, 50mM KCl, 10mM Tris HCl (PH 8.3), 2.5mM $MgCl_2$, 0.001% gelatin, 10% DMSO and 1.25 units of Ex Taq polymerase (Takara) with proof-reading activity. Amplification was performed with denaturation at 95°C for 2 min, 35 cycles of denaturation-annealing-extension at 95°C for 1 min, 56°C for 1 min, 72°C for 2 min, and extension at 72°C for 5 min. One μL of the first round product was used for the second round of PCR with the same PCR reaction mixture except 1.0μM each of inner sense primer F3897 and antisense primer R5181. Amplification conditions for the second round were the same as those for the first round. Ten μL from the second round of PCR products were analyzed by 8% polyacrylamide gel electrophoresis and stained with ethidium bromide.

To avoid contamination of PCR products, all reagents were UV-irradiated and ART aerosol resis-

tant pipette tips (Molecular Bio-Products, Inc., San Diego, CA) were used. Samples from healthy persons were always used as negative controls. In all of the experiments, the negative samples showed negative results for HAV RNA.

Direct Sequencing of HAV cDNA Fragments

For sequencing the region from nucleotides 3897 to 5181 including 2C between nucleotides 3996 and 5000 of HAV, 20μL of the nested PCR products was centrifuged in microconcentrators (Ultrafree C3KT, Japan Millipore Ltd., Tokyo, Japan). The concentration of the PCR products was determined by optical densitometry (Ultrospec III, Amersham Pharmacia Biotech, Buckinghamshire, England), and a volume of product equivalent to 130ng was used for single-sided amplification with the inner sense or antisense primers with a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) per the manufacturer's instructions. The dye terminator products were purified over Centri-Sep spin columns (Applied Biosystems, Foster City, CA). Direct sequencing was performed with an ABI 377 DNA sequencer (Applied Biosystems).

Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported herein appear in DDBJ/EMBL/GenBank nucleotide sequence databases with the following accession numbers: AB082174 for A1, AB082130 for A5, AB082131 for A165, AB0821323 for A204, AB082133 for A205, AB082134 for A206, A082135 for A414, AB082136 for A502, AB082137 for A601, AB082138 for A6, AB082139 for A159, AB082140 for A160, AB082141 for A161, AB082142 for A196, AB082143 for A197, AB082144 for A200, AB082145 for A302, AB082146 for A702, AB082147 for A811, AB082148 for A7, AB082149 for A9, AB082150 for A20, AB082151 for A28, AB082152 for A32, AB082153 for A51, AB082154 for A68, AB082155 for A75, AB082156 for A77, AB082157 for A80, AB082158 for A85, AB082159 for A157, AB082160 for A162, AB082161 for A168, AB082162 for A195, AB082163 for A207, AB082164 for A301, AB082165 for A303, AB082166 for A304, AB082167 for A306, AB082168 for A307, AB082169 for A404, AB082170 for A406, AB082171 for A712, AB082172 for A713, AB082173 for A814.

RESULTS

Clinicopathological Characteristics of Patients Analyzed for HAV 2C

Characteristics of 45 patients with hepatitis A analyzed for HAV 2C at admission are summarized in **Table 2.** None of the cases were associated with an epidemic.

Differences in mean age between severe (FH and AHs) and non-severe cases (AH) were not statistically significant ($p=0.08$). Serum was sampled 2 to 17 days after clinical onset. Mean ALT level was higher in FH and AHs than in AH ($p<0.001$). Mean prothrombin time was prolonged in FH and AHs compared with that in AH ($p<0.001$). Mean total bilirubin level and mean IgM-HA titer were not significantly different between severe and non-severe cases ($p=0.23$ and $p=0.17$, respectively) **(Table 2)**.

Four of 9 patients with FH died of hepatic failure,

TABLE 2 Clinical and Laboratory Data of 45 Patients

	FH	AHs	AH
n	9	10	26
Recovery/death	5/4	8/2	26/0
Sex (M/F)	5/4 [1]	10/0 [1]	16/10 [1]
Age*	45.6±12.0 [2]	40.1±11.7 [2]	36.9±10.4 [2]
PT (%)*	14±7 [3]	32±8 [3]	67±17 [3]
ALT (IU/L)*	5818±3480 [4]	5317±1847 [4]	3196±1752 [4]
T-Bil (mg/dL)*	10.5±7.4 [5]	4.3±3.1 [5]	5.3±3.1 [5]
IgM-HA (cut-off index)*	4.3±1.6 [6]	4.6±1.3 [6]	5.3±2.0 [6]

*Mean ±SD; [1] statistically not significant between FH+AHs and AH; [2] statistically not significant between FH+AHs and AH; [3] statistically significant ($p<0.0001$) by student's t test between FH+AHs and AH; [4] statistically significant ($p=0.002$) by Welch's test between FH+AHs and AH; [5] statistically not significant between FH+AHs and AH; [6] statistically not significant between FH+AHs and AH.

TABLE 3 Nucleotide and Amino Acid Differences of HAV 2C between HM175 and Each Strain

	% nucleotide identity	% amino acid identity
FH (n=9)	89.0	97.6
A1	90.0	97.9
A5	88.3	97.0
A165	88.5	97.6
A204	88.9	97.3
A205	90.0	97.9
A206	88.6	97.3
A414	88.9	97.3
A502	88.8	97.6
A601	89.3	98.2
AHs (n=10)	88.6	96.5
A6	88.3	97.0
A159	89.9	97.3
A160	88.9	96.4
A161	88.8	97.9
A196	86.5	91.6
A197	89.0	97.9
A200	88.8	97.3
A302	88.3	96.1
A702	89.3	97.6
A811	88.5	95.8
AH (n=26)	89.0	96.8
A7	95.9	98.8
A9	88.7	96.7
A20	89.1	97.6
A28	89.1	97.6
A32	88.8	97.3
A51	88.5	97.6
A68	89.3	97.9
A75	88.3	97.3
A77	89.2	97.9
A80	89.1	97.6
A85	88.6	97.0
A157	89.5	97.3
A162	88.5	96.4
A168	88.5	96.4
A195	88.8	97.6
A207	85.9	90.1
A301	88.3	96.7
A303	89.2	96.4
A304	88.6	96.4
A306	87.9	97.3
A307	88.7	96.7
A404	89.7	97.6
A406	89.3	96.7
A712	88.9	92.8
A713	88.8	97.3
A814	88.3	97.3

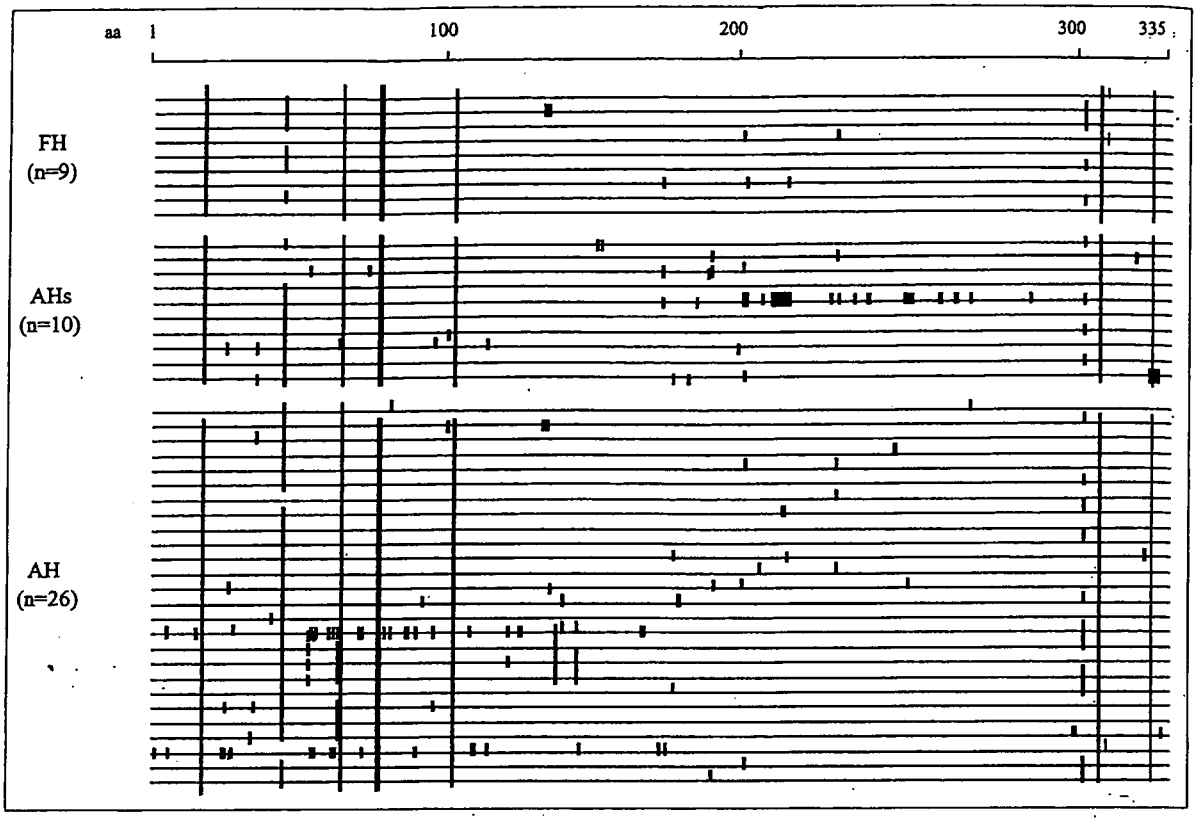

FIGURE 1 The distribution of amino acid variations of 2C of HAV is schematically shown. Horizontal lines indicate identical amino acids between the individual strains and HM-175. Vertical lines indicate positions where amino acid changes occurred in the individual strains compared to HM-175. FH consisted of A1, A5, A165, A204, A205, A206, A414, A502, and A601, respectively. AHs consisted of A6, A159, A160, A161, A196, A197, A6, A200, A302, A702, and A811, respectively. AH consisted of A7, A9, A20, A28, A32, A51, A68, A75, A77, A80, A85, A157, A162, A168, A195, A207, A301, A303, A304, A306, A307, A404, A406, A712, A713, and A814, respectively.

and 2 of 10 patients with AHs died of sepsis. All 9 FH cases needed artificial liver support (plasma exchange and hemodiafiltration). Seven (16%) patients - 3 (33%) with FH, one (10%) with AHs, and 3 (12%) with AH - had acute renal failure and were treated by hemodiafiltration.

Two patients with AH were positive for HBsAg and anti-HBe antibody, and 1 patient with AH was positive for anti-nuclear antibody, but they showed typical hepatitis A courses. IgM-EBV, IgM-HSV, IgM-CMV, anti-nuclear antibody, anti-smooth muscle antibody, liver kidney microsomal antibody-1, and anti-mitochondrial antibody were negative in all examined cases of FH and AHs. One FH patient and one AHs patient had histories of heavy alcohol consumption. One male patient with AH was homosexual.

Histological examination was performed in all 9 FH cases, 6 of 10 AHs cases, and 9 of 26 AH cases in the convalescent phase or post-mortem. In the FH cases, liver histology revealed massive necrosis in 3 patients, and submassive necrosis in 2. Submassive necrosis was found in the 2 AHs cases that died of sepsis. Liver histology in the 2 patients with histories of heavy alcohol consumption showed pericellular fibrosis, consistent with alcoholic liver disease. The histological findings of the other cases showed acute hepatitis to be in a residual phase or subsiding.

Sequencing Analysis of HAV Strains from FH, AHs, and AH

Sequences of 2C between nucleotides 3996 and 5000 of HAV were determined in the 45 patients, and compared with wild-type HAV strain HM-175.

Nucleotide sequences of 2C had homology of 89.0±0.6% in FH, 88.6±0.9% in AHs, and 89.0±1.6% in AH, with the differences not being statistically significant. Deduced amino acid sequences had homology of 97.6±0.4% in FH, 96.5±1.9% in AHs, and 96.8±1.7% in AH. The difference between FH and AH was statistically significant (p=0.04) **(Table 3)**.

The distribution of amino acid variations is shown schematically in **Figure 1**. Throughout the entire 2C gene, no specific substitutions of amino acids residues were found among the three groups.

Substitution Sites in the Proposed NTP Binding Motif

HAV 2C contains a nucleoside-triphosphate (NTP)-binding motif. This motif consists of elements "A" (2/5 hydrophobic stretch) G/AXXGXGKS/T, where X stands for any amino acid, and "B" (3/5 hydrophobic stretch) D or DD/E. The residues mutated within the conserved A and B sites of the NTP-binding motif of 2C are critical in RNA replication and virus proliferation.