Table 5. Characteristics of the four patients who were infected with HBV of subtype A′

Features	Patient 1	Patient 2	Patient 3	Patient 4
Sex	M	F	F	M
Age (years)	26	27	43	47
Family history		Father Brother		
Diagnosis	ASC	CH	CH	CH
Liver histology	A0/F0	A1/F1	A1/F1	A1/F2
HBV DNA (LGE/ml)	<3.7	≥8.7	<3.7	≥8.7
HBeAg	±	±→±	±	±→±
Nucleotide 1858	C	C	T	C
Wild-type				
Core promoter	No	Yes	Yes	No
Precore sequence	Yes	Yes	Yes	Yes

A, grade of necroinflammatory activity; F, stage of fibrosis; LGE, log genome equivalent

Discussion

In Japan, by far the greatest number of carriers contracted their HBV infection perinatally through mother-to-bady transmission decades ago.[15,16] By contrast, individuals in Europe and the United States acquire HBV infection in adulthood. There are remarkable differences in the distribution of HBV genotypes between Japan and western countries. Genotypes B and C are prevalent in Japan, in contrast to genotypes A and D common in European countries,[4,5] while all seven HBV genotypes are found in the United States, with the distribution dependent on the ethnicity of carriers.[17]

We previously reported that the proportion of patients with acute hepatitis infected with HBV/A in the Tokyo metropolitan area was significantly higher ($P < 0.0001$) than that in those with chronic hepatitis who visited the same hospital.[18] In the present study, a mother with HBV infection was not reported by any of the patients infected with HBV/A who presented with acute hepatitis. Only one patient infected with HBV/A, with chronic hepatitis, was found to have a mother with HBV infection. Her genotype was B, however, thereby excluding mother-to-baby transmission in this patient. These results indicate that, at least in Tokyo, most infection with HBV/A occurs horizontally rather than perinatally, in corroboration with our previous findings.[18]

It may be surprising that 5 of the 26 (19%) patients with acute hepatitis who were infected with HBV/A failed to clear infection. They had all contracted de-novo HBV infection, because they did not test positive for HBsAg in health check-ups before they came down with acute hepatitis. In Japan, acute HBV infection in adulthood evolves into chronicity in only fewer than 1%, unlike that in western countries, where it persists in approximately 10%.[19] It appears that primary infection with HBV of genotype A would tend to be chronic more frequently than those of the other genotypes, even when it is contracted in adulthood.

There is evidence in support of a role of homosexuality in HBV/A infection. A cluster of HBV/A strains was identified in men having sex with men in Amsterdam.[20] In the present study, the most common route of infection with HBV/A in patients with acute hepatitis was homosexual activity, as reported by 11 of 28 patients (39%). HBV/A infection persisted in 4 of the 11 (36%) who had contracted infection by homosexual contacts. There are increasing numbers of young male homosexuals in the Tokyo metropolitan area, some of whom may be at increased risk of infection with HBV/A as well as human immunodeficiency virus type 1.[21] It is worrying that the persistence of acute HBV/A infection will increase in Japan in the future. For preventing the chronicity of HBV/A infection, hepatitis B vaccine would be effective in persons at risk of contracting infection by sexual contacts, in particular.

HBV/A isolates have the nucleotide 1858 of C (C1858) that makes a pair with T1896. Therefore, the G-to-A point mutation in the precore region creating the stop codon is prevented in HBV/A isolates, because A1896 is incompatible with C1858 and destroys the conformation of the pregenome encapsidation signal.[9,10] Hence, the precore mutation (A1896) in HBV/A isolates always accompanies C-to-T mutation at nt 1858. T1858 in HBV/A infection was detected in 5 of the 14 (36%) asymptomatic carriers (all of whom were without serum HBeAg), this being significantly more frequent than in none of the 21 patients in whom acute hepatitis had resolved ($P = 0.008$); 20 of them (95%) presented with HBeAg in serum.

HBV/A isolates in South Africa are phylogenetically different from those in Europe and the United States, and most of them are of subtype A′.[7,22] Subtyping of HBV/A isolates into A (the original genotype A) and

A′ was feasible in 58 (85%) of the 68 patients in the present study. The subtype was A in 53 of these 57 (93%) HBV/A isolates, and A′ in the remaining 4. Where the HBV/A strains of subtype A′ came from, and how they have spread in Tokyo, is not certain, and these questions need to be sorted out in future epidemiological studies. All HBV/A infections of subtype A′ occurred in persistent HBV infection and occurred in 1 asymptomatic carrier and 3 patients with chronic hepatitis, of whom 1 cleared infection during 3 years of follow-up.

How these four patients contracted HBV infection of subtype A′ is a matter of conjecture. Although they all had chronic HBV infection, a possibility remains that there had been evolution of acute infection with subtype A′, which may have been acquired through sexual contacts. Special care will have to be devoted to them, because infection with subtype A′ in Africa is associated with a high incidence of hepatocellular carcinoma,[23] although its development would be accelerated by afratoxin there.

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〔原　著〕

肝検診地域の住民におけるC型肝炎ウイルス感染感受性遺伝子の解析

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要　旨

C型肝炎ウイルス（HCV）感染症において，HCV感染後の経過は個人間で多様である。HCVに対する感染感受性の相違に関わる宿主の遺伝的要因を解明する目的で，HCVに感染した一般住民の遺伝子一塩基多型（SNP）を網羅的に解析した。対象は，HCV高感染地域において肝臓病検診を受診した住民で，遺伝子解析の同意の得られた675名である。HBs抗原陰性かつ抗ウイルス治療を受けていないHCV抗体陽性者は238名であった。HCV抗体陽性者において，持続感染者（189名）と既往感染者（49名）の二群間で遺伝子SNPを解析した結果，10遺伝子における12SNPの出現頻度に有意差（$P<0.05$）が認められた。これらは，HCV感染に際し，ウイルスの細胞表面への接着と細胞内増殖，そして免疫担当細胞の働きに関わる可能性がある遺伝子である。今回の研究で検出された遺伝子群は，HCV感染感受性に関わる候補遺伝子であり，HCV感染症における機能解析が今後の重要な研究課題である。

キーワード　HCV，SNP，検診

【はじめに】

現在，わが国におけるC型肝炎ウイルス（HCV）感染者は200万人以上に上るとも推定されている。また，慢性肝疾患の大多数はHCV持続感染に起因するものであり，肝細胞癌患者の70%以上にHCV感染が見られる[1)]。したがって，HCV感染者の早期発見とその治療対策は，急務である。平成14年度より，老人保健法による住民検診において，HCV感染検査が節目検診あるいは節目外検診として施行されるようになり，国を挙げてのHCV感染対策が始まっている。HCV感染者数の分布には地域的に偏りがあり，本邦において，それは西高東低である。山形県においても，HCV感染者の分布には，地域間に偏りがあり，高感染地域が存在する。私達は，HCV高感染地域における肝臓病検診の重要性を考え，HCV感染者の多い山形県R町において，肝臓病検診事業を1991年度より継続して行っている[2)]。ヒトゲノムが解明され，疾患における遺伝的要因の解明が急速に進みつつある。感染症の領域においても，宿主側の感染感受性に関わる遺伝的要因の解明は，その感染症の病態解明に寄与するとともに，新たな予後予測因子や治療薬の開発にとって重要である。HCV感染後の経過は個人間で多様であるが，感染後の経過に影響する宿主側の遺伝的要因については未解明である。そこで今回，肝臓病検診を継続的に実施しているR町において，HCV抗体陽性の住民におけるウイルス持続感染者と既往感染者の二群間で，遺伝子一塩基多型（Single nucleotide polymorphism：SNP）の網羅的な解析を行った。本研究により，HCV感染後の経過に影響を与える個人の遺伝子多型性が明らかとなり，将来それら遺伝子群の機能解析により，C型肝炎の病態解明，あるいは新しい診断法や治療法の開発に結びつくことが期待される。

【対象と方法】

対 象

山形県R町はHCV感染者が多い地区である。私達は1991年から95年度までに，検診の同意が得られた6歳以上の住民7925名について，HCV感染調査を行い，HCV抗体陽性者が1078名（13.6%），そのうちHCV RNA陽性者が846名に上ることを確認した。その中でも47集落より形成されるR町北部地区における住民のHCV抗体陽性率は特に高く，6歳以上の住民で19.5%（602/3094名），40歳以上の住民で32.4%（584/1805名）であった[2]。HCV RNA陽性者については，96年以降も毎年，追跡検診を行っており，本コホート研究によるHCV RNA陽性者846名からの肝細胞癌の発生は，10年間で21例（2.5%）であった[3]。HCV感染者が多く集積し，感染者の病歴が十分把握されているR町北部地区を中心に，2001年度に再度，20歳以上の一般住民1040名を対象として，採血によるHCV感染調査と肝機能検査を行い，遺伝子解析の同意が得られた被験者には遺伝子SNP解析を行った。対象者は全員が日本人であり，外国からの移住者はいなかった。本研究は，2001年度において，国が定めた「ヒトゲノム，遺伝子解析研究に関する倫理指針」に則り，山形大学医学部倫理委員会の承認を得て開始された。

方 法

1．受診者の問診および本研究に関する同意の取得

検診受診者について，抗HCV治療としてのインターフェロン（IFN）治療歴の有無について問診を行った。また，遺伝子検査に関する同意について，当大学医学部倫理委員会で認められた所定の文書で取得した。

2．肝炎ウイルス感染検査および肝酵素検査

肝炎ウイルス感染検査は，HCV感染歴の有無を調べる目的でHCV抗体検査（HCV EIAII：Abott社製），および現在のウイルス血症の有無を調べる目的でHCV遺伝子増幅法によるHCV RNA定性検査（Amplicor HCV v2.0：Roche Diagnostics社製）を行った。またHBs抗原の測定（HBsAg Dainapac：Dainabot社製）を行った。肝酵素検査については，alanine aminotransferase（ALT）値（正常値：30U/ml未満）を測定した。

3．遺伝子SNP解析

遺伝子SNP解析の同意が得られた被験者の全血10mlより，ゲノムDNAをDNA抽出用Kit（Talent社製）を用いて抽出した。SNP解析は，Pre-Development Taqman Assay Regents for Allelic Discrimimation Kit（Applied Biosystems社製）を用いて，TaqMan PCR法にて行った[4]。解析した遺伝子は1784遺伝子であり，日本人のSNPデータベースであるJSNPデータベースより，4784 SNPを抽出し，網羅的な遺伝子多型性解析に供した。これらのSNP解析は，ヒュービットゲノミクス社（東京都千代田区）にて行われた。

4．ケースコントロール解析

本研究において，HCVに対する宿主の感染感受性に影響を与える遺伝的要因を明らかにするため，HCV感染が成立したと考えられるHCV抗体陽性者について，ウイルス血症の有無についてのケースコントロール研究を設定した。即ち，HCV抗体陽性者について，HCV RNA陽性者（持続感染者）とHCV RNA陰性者（既往感染者）の二群に分け，両群間における遺伝子多型の解析を行った。本解析には，上述の1784遺伝子より，HCVの感染成立や免疫に関与すると考えられる103遺伝子を絞り込み，これら遺伝子の269SNPについての検討を行った。

5．統計解析

臨床疫学データの統計解析には，Mann-Whitney U testおよびFisher's exact testを用いた。遺伝子SNP解析における統計解析は，二群間におけるSNP出頻頻度を，Fisher's exact testにて検定した。$P<0.05$を有意差ありとした。

図1：被験者のフローチャート

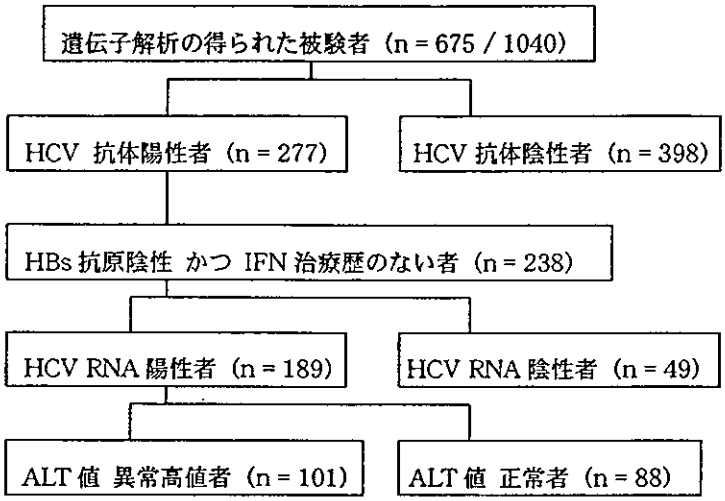

表1：SNP研究群の特性

	血清 HCV RNA		
	陽性者（n = 189）	陰性者（n = 49）	*P*
性別（男性）	70	17	0.45
年齢（歳）	66.9 ± 9.5	66.6 ± 9.4	0.84
HCV 抗体陽性	189	49	
ALT (U/ml, mean ± SD)	43.7 ± 43.8	21.9 ± 17.7	< 0.001

表2：HCV感染者におけるHCV感染感受性候補遺伝子とSNP

Gene symbol	SNP	At-risk allele	Genetic model	*P* value	odds ratio (95% CI)	SNP ID (IMS-JST)
SULT2B1	A/G	G	dominant	0.0200	5.23 (1.35-20.27)	075438
AP1B1	C/T	T	dominant	0.0150	2.31 (1.21-4.41)	020466
	C/T	T	dominant	0.0150	2.30 (1.20-4.38)	020470
IFNAR2	A/C	A	recessive	0.0220	2.31 (1.15-4.55)	056061
IFI27	A/G	A	dominant	0.0230	2.18 (1.15-4.14)	010856
IFI41	C/T	T	dominant	0.0350	4.14 (1.15-14.92)	013416
PRL	A/G	G	dominant	0.0030	2.75 (1.40-5.40)	055776
CD4	C/T	C	dominant	0.0180	2.71 (1.22-6.02)	006379
TGFB1	G/T	T	dominant	0.0140	2.42 (1.23-4.75)	013663
	G/T	G	dominant	0.0490	2.13 (1.06-4.30)	013665
LTBP2	A/G	G	dominant	0.0006	3.83 (1.81-8.11)	058392
TNFRSF1A	G/T	G	dominant	0.0490	2.04 (1.06-3.94)	034139

【結 果】

1．HCV感染と血清ALT値

被験者1040名中，遺伝子検査の同意を得られた者は675名（同意取得率：65%）であった。これら675名の検診結果をフローチャートとして（図1）に示す。HCV抗体陽性者は277/675名（41.0%）であった。遺伝子解析の対象となるHCV抗体陽性者は，このうちHBs抗原陽性者および問診により抗ウイルス療法を受けた者を除外し，238名であった。HCV RNA定性検査では，陽性が189名（79.4%），陰性が49名（20.6%）であった。またHCV RNA陽性の持続感染者における血清ALT値を検討したところ，ALT値が異常高値（30U/ml以上）であった者が101名（53.4%），正常値（30U/ml未満）であった者が88名（46.6%）であった。

2．HCV持続感染者と既往感染者における臨床疫学データの検討

本研究におけるSNP研究群の特性を（表1）に示す。HCV抗体陽性者（238名）におけるHCV RNA陽性者（189名）とHCV RNA陰性者（49名）の二群間において，男女比や年齢に有意差はなかった。年齢は両群において平均約66歳とほぼ等しかった。血清ALT値は，HCV RNA陽性者ではHCV RNA陰性者に比し，有意に高値であった（43.7±43.8U/ml vs 21.8±17.7U/ml：P<0.001）。

3．HCV感染者におけるHCV感染感受性候補遺伝子の検討

HCV抗体陽性者におけるHCV RNA陽性者とHCV RNA陰性者間の遺伝子SNPの出現頻度を比較検討することで，HCVの持続感染や排除に関わる遺伝子候補群が検出された。結果を（表2）に示す。

（1）HCVの細胞表面への接着に影響する遺伝的要因

HCVの感染標的となる細胞表面への接着は，持続感染の維持に重要である。HCVが属するフラビウイルスは，細胞表面への接着に際し，細胞表面上のヘパリンやヘパリン硫酸などの硫酸多糖や接合蛋白（adaptor protein）に接合する。本研究により，これらの産生に関与が示唆されるSULT2B1遺伝子，AP1B1遺伝子におけるSNP出現頻度において，HCV RNA陽性者とHCV RNA陰性者の二群間において有意差が認められた。

（2）HCVの細胞内増殖に影響する遺伝的要因

HCVの細胞内複製阻害に際し，ウイルス感染細胞や免疫担当細胞で産生されるIFNは抗ウイルス蛋白として最も重要なものの一つである。本研究において，IFN関連遺伝子として，IFNAR2遺伝子，IFI27遺伝子，IFI41遺伝子におけるSNP出現頻度において，HCV RNA陽性者とHCV RNA陰性者の二群間において有意差が認められた。

（3）HCVの感染免疫に影響する遺伝的要因

HCVの持続感染や排除に関係する免疫系に関与する遺伝的要因は重要である。本研究により，PRL，CD4，TGFB1，LTBP2，TNFRSF1の各遺伝子におけるSNP出現頻度において，HCV RNA陽性者とHCV RNA陰性者の二群間において有意差が認められた。

【考 案】

感染症において，病原体に対する宿主の免疫応答は個人間で多様である。特に，HCV感染症においては，ウイルス感染が急性期に治癒する者から慢性化する者，慢性化した場合でも健康保因者にとどまる者から幾多の程度の活動性肝炎に至る者まで，その感染史は個人間で多様である。個人間における遺伝的要因がHCV感染の成立とその後の免疫応答の差異に関わっているが，HCV感染宿主における遺伝子多型性についてはほとんど解明されていない。本研究において，私達はC型肝炎の地域住民コホート研究を発展させ，住民の協力を得て臨床疫学データと遺伝子サンプルを採集した。そして匿名化されたサンプルを用いて，ヒトゲノム上に見いだされているSNPを解析する

ことで，HCVの感染感受性に影響すると思われる宿主の遺伝的要因を見出すことを試みた。

これまで，私達は，山形県におけるHCVの高感染地区において，1991年度より現在に至るまで，住民肝臓病検診を継続して行い，C型肝炎をターゲットとしたコホート設定とその追跡調査を行ってきた。住民検診は，大学，自治体および住民が一体となり行われている。住民検診の結果は，受診者へ通知され，HCV抗体陽性者に対しては肝臓病に対する治療のアドバイスが行われてきた。また，地域住民には講演会を通してC型肝炎に対する啓蒙活動を行ってきた。本コホート研究により，一般のHCV感染住民におけるHCV RNA自然消失率や発癌率等のHCV感染症の自然史の一端が解明されている[3)5)6)]。このような研究基盤のうえに，HCVの感染後経過に影響する宿主の遺伝的要因を明らかにする目的で，分子疫学研究を行うことは意義深いものと思われる。

HCVの感染後経過に影響する宿主の遺伝的要因を解明するために，今回の検討では，HCV抗体陽性者におけるHCV RNA陽性者とHCV RNA陰性者の二群間にみられる遺伝的多型性を，遺伝子SNP出現頻度の違いから明らかにした。このようなケースコントロールの設定により，HCV感染後のウイルス持続感染と排除に関与する遺伝的要因の解明が期待される。今回の研究では，HCV RNA陽性の持続感染者は189/238名（79%），HCV RNA陰性の既往感染者は49/238名（21%）であり，約70%から80%とされるHCV感染のキャリア化率[7)]にほぼ一致した。また，両群の平均年齢が約66歳でほぼ同一であることを考え併せると，両群の被験者は過去のほぼ同一時期にHCVに感染したものと思われる。以上から，本研究における被験者の臨床疫学データと遺伝子サンプルは貴重であると思われる。

HCV感染における持続感染者と既往感染者の遺伝子SNP解析により，①HCVの感染標的細胞上におけるウイルス接着に関わる遺伝子多型性，②ウイルスの細胞内増殖に関わる遺伝子多型性，③免疫担当細胞に関わる遺伝子多型性，が明らかとなった。HCVの感染標的細胞への感染成立のメカニズムについては，そのレセプターの同定を含め，未解明である。HCVが属するフラビウイルスの標的細胞への感染経路については，ウイルスの細胞膜への融合に際し，ウイルス粒子の細胞膜表面上における硫酸多糖への捕捉と，接合蛋白によるウイルスの被覆が必要である[8)9)]。最近，HCV感染に際しても，HCVの細胞表面への吸着に際し，ヘパラン硫酸多糖が必要であることが報告されている[10)]。その後ウイルスはレセプターへ吸着し，感染が成立することが知られている。本研究にて，HCV持続感染者と既往感染者の間には，硫酸多糖および接合蛋白の産生に関与する2種の遺伝子，SUT2B1およびAP1B1に遺伝子多型性が存在することが明らかとなった。これは，細胞表面上のHCV接着因子の発現に個人間における差異がある可能性を示唆している。また，IFNは，HCV感染細胞や免疫細胞で誘導される重要な抗ウイルス蛋白である。IFNの標的細胞におけるシグナル伝達は，ウイルスの複製阻害，宿主の自然免疫や細胞性免疫の賦活などの広範な抗ウイルス作用を惹起する[11)]。HCV持続感染者と既往感染者の間に認められたIFNAR2，IFI27，IFI41などの遺伝子多型性は，個人間におけるIFN感受性に差異がある可能性を示唆している。さらにHCV感染においては，T細胞，マクロファージあるいはNK細胞等の各種の免疫担当細胞により，多くのサイトカインネットワークが存在する。特にCD4陽性細胞はHCV感染者の肝障害時の免疫調節作用に重要な役割を担っている[11)]が，HCV持続感染者と既往感染者間にはこのCD4遺伝子のSNP出現頻度に違いが認められた。これら，HCV感染に深く関与すると考えられる免疫担当細胞に影響を与えると考えられる，PRL，TGFB1，LTBP2，TNFRSF1等の遺伝子にも遺伝子多型性が見出された。以上より，これらの遺伝子多型性は，HCVの感染宿主におけるウイルスの持続感染と排除に関わる免疫学的に重要な遺伝的要因である可能性がある。今後は，これら遺伝子のHCV感染症における機能解析が重要であ

る。

【結 語】

HCV高感染地域における住民を対象として，HCVの感染後の経過に影響する遺伝的要因の解明を行った。本研究で検出された遺伝子多型を有する遺伝子群は，HCV感染者のウイルス持続感染と排除に影響する候補遺伝子であり，今後，これら遺伝子のHCV感染における機能解析により，HCVトランスレーショナル・リサーチの発展が期待される。

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Analysis of genetic polymorphisms in individuals infected with hepatitis C virus (HCV) in an area endemic for HCV infection

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The outcome of hepatitis C virus (HCV) infection varies among individuals. To elucidate the host genetic factors involved in the outcome, we conducted a population-based association study in which 238 individuals positive for anti-HCV antibody were genotyped for single nucleotide polymorphisms (SNPs) in the selected candidate genes. Those were seronegative for HBs antigen and had no history of anti-viral therapy. Allelic and genotypic analyses of SNPs were performed between the subjects with and without viremia. Twelve SNPs in 10 genes were listed as candidate genetic polymorphisms that might influence the viremia status. These may reflect the genetic variations in HCV-infected individuals that are associated with susceptibility to infection, viral replication and immunity against HCV. It is important to define the function of these genes with regard to HCV infection.

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Genetic variations in humans associated with differences in the course of hepatitis C☆

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Abstract

The outcome of hepatitis C virus (HCV) infection varies among individuals, but the genetic factors involved remain unknown. We conducted a population-based association study in which 238 Japanese individuals positive for anti-HCV antibody were genotyped for 269 single nucleotide polymorphisms (SNPs) in 103 candidate genes that might influence the course of infection. Altogether, 50 SNPs in 32 genes were listed. Genetic polymorphisms in IL4, IL8RB, IL10RA, PRL, ADA, NFKBI, GRAP2, CABIN1, IFNAR2, IFI27, IFI41, TNFRSF1A, ALDOB, AP1B1, SULT2B1, EGF, EGFR, TGFB1, LTBP2, and CD4 were associated with persistent viremia ($P < 0.05$), whereas those in IL1B, IL1RL1, IL2RB, IL12RB1, IL18R1, STAT5A, GRAP2, CABIN1, IFNAR1, Mx1, BMP8, FGL1, LTBP2, CD34, and CD80 were associated with different serum alanine aminotransferase levels in HCV carriers ($P < 0.05$). The sorted genes allow us to draw novel hypotheses for future studies of HCV infection to ultimately identify bona fide genes and their variations.
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Keywords: Hepatitis C virus; HCV RNA; Single nucleotide polymorphism; SNP; Infection; Genetic factor; Immune response

Hepatitis C virus (HCV) is an important human pathogen that causes acute and chronic liver diseases worldwide. There are an estimated 170 million HCV carriers, rendering it a major public health problem [1]. Most patients with HCV infection fail to clear the virus and develop chronic hepatitis with a risk of progression to cirrhosis or hepatocellular carcinoma [2]. However, a small proportion of patients are known to show resolution of the infection in a self-limiting manner [3,4]. The immune response of each individual against HCV in the acute phase is likely to account for this difference [5]. Genetic factors attributed to each individual may play an important role in HCV infection and the subsequent immune responses, but these host genetic factors have not been elucidated.

We have been following a cohort of 1040 inhabitants in Japanese hamlets where hepatitis C is endemic. Our previous study [6] showed that hepatitis C was endemic in the northern district of one rural town, including the hamlets observed in this study, the overall positivity rate of anti-HCV antibody being 19.5% (602/3094) in inhabitants aged six or over. The positivity rate of anti-HCV antibody increased with age in this area, and particularly showed a marked difference between subjects younger than 40 years (18/1289; 1.4%) and those aged 40 or over (584/1805; 32.4%). Although HCV

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infection is hyperendemic in several areas in Japan [6–9], the routes of HCV transmission remain unknown in most cases. Recurrent use of syringes and needles [7], folk remedies such as acupuncture [8] may be a factor in community-acquired HCV infection. The routes of HCV transmission are still obscure in this community. All inhabitants are Japanese in this local area, and movement of people in and out of the region has been rare for decades. This area provided an excellent setting for investigating genetic background, because stratification of the population and the resulting tendency to detect a false-positive association could be minimized.

We carried out a population-based, molecular epidemiological study by investigating the presence of single-nucleotide polymorphisms (SNPs) in the inhabitants infected with HCV in this area. In this paper, we report on the candidate SNPs and genes that may lead to the identification of important genetic variations associated with differences in the course of HCV infection.

Materials and methods

Subjects. During a local community health examination of the area, 675 adult individuals aged 20 or over (286 men and 389 women, aged from 20 to 88 years [mean ± SD, 57.6 ± 13.5 yr]) were recruited for blood tests including measurements of levels of alanine aminotransferase (ALT), anti-HCV antibody, serum HCV RNA, and for their genetic variations, SNPs. The present study was approved by the Ethical Review Committee of Yamagata University, and written informed consent was obtained from all the subjects recruited. The presence of anti-HCV antibody and serum HCV RNA status was examined using an enzyme immunoassay kit (HCV EIAII Abbott; Abbott Japan, Tokyo, Japan) and the Amplicor HCV RNA detection kit (Amplicor HCV v2.0; Roche Diagnostics, Tokyo, Japan), respectively. This Amplicor HCV RNA assay has a lower limit of detection of 50 IU/ml, and thus could be used to determine if HCV viral clearance had occurred. HCV is considered cleared if virus levels fall below 50 IU/ml of HCV RNA/ml [10]. The normal level of ALT was defined by analyzing the level of healthy subjects in this area, all of whom were seronegative for both hepatitis B surface antigen and anti-HCV antibody, and were non-habitual drinkers of alcohol. Percentiles smaller than 2.5 and larger than 97.5 were excluded. The normal ALT level in this study was then defined as an ATL value < mean value + 2 standard deviation (SD) (ALT reference, upper limit of normal: 29 IU/L). The survey revealed that 277/675 (41.0%) of subjects were positive for anti-HCV antibody, confirming that there was an abnormally high incidence of HCV infection history. After excluding subjects with a history of anti-viral therapy using interferon, or those with positive hepatitis B surface antigen, 238 (35.3%) subjects (87 men and 151 women, aged from 32 to 88 years [mean ± SD, 66.8 ± 9.4 yr]) positive for anti-HCV antibody were enrolled in the study and tested for serum HCV RNA. All the enrolled subjects were retested for serum HCV RNA over a minimum of 12 months, using serum samples stored at −80 °C that had been collected at the last follow-up study, and their status of serum HCV RNA was defined. These subjects were classified into those with HCV viremia (PP group, $n = 189$; 70 men and 119 women, aged from 32 to 88 years [mean ± SD, 66.9 ± 9.5 yr]) and those without HCV viremia (PN group, $n = 49$; 17 men and 32 women, aged from 38 to 79 years [mean ± SD, 66.6 ± 9.4 yr]). The individuals in the PP group were further divided into two categories: those with a high ALT level over the upper limit of normal (ALT ≧ 30 IU/L; range: 30–351 IU/L [mean ± SD, 63.7 ± 30.4 IU/L], $n = 101$) and those with a low ALT level within the normal range (ALT < 30 IU/L; range: 7–29 IU/L [mean ± SD, 20.8 ± 4.2 IU/L], $n = 88$). The mean ALT level for the above two categories was significantly different (63.7 ± 30.4 IU/L vs. 20.8 ± 4.2 IU/L; $P < 0.001$, Mann–Whitney U test).

Selection of SNPs. To elucidate the genetic variations associated with differences in the course of HCV infection in individuals, an intensive analysis was undertaken of SNPs in the genes of all 238 subjects. The selected genes were classified into the following categories: (1) cytokine and immune system-related genes; (2) viral infection-related genes; (3) liver function-related genes; and (4) CD series genes. The gene list was not necessarily a thorough one for several reasons: (1) it was not possible to extract all of the genes potentially relevant to HCV infection; (2) some of the genes initially listed did not have SNPs on the database; and (3) some of the SNPs did not make good assays and were excluded from the initial list. SNPs from the genes were further selected from the database of JSNPs, which contains SNPs that have been identified in the Japanese population (http://snp.ims.u-tokyo.ac.jp/index_ja.html). When choosing multiple SNPs from one gene, we attempted to make selections that were evenly spaced. Table 1 shows the final list of 103 candidate genes and 269 SNPs that were selected and assayed.

Genotyping of polymorphisms. Genomic DNA was extracted from 10 ml of whole blood with a kit (Talent, Trieste, Italy). The SNP typing was conducted with a fluorogenic polymerase chain reaction (PCR)-based technique [11] using Pre-Developed Taqman Assay Reagents for Allelic Discrimination (Applied Biosystems, Foster City, CA), and allele frequencies were determined. The PCR for detection of SNPs was carried out according to the manufacturer's instructions. Briefly, 3 ng of DNA was mixed with 10× Allelic Discrimination Assay Mix (900 nM each forward and reverse primer, 200 nM each reporter dye [FAM or VIC]-labeled probe) and 2 × TaqMan Universal PCR Master Mix (Applied Biosystems). The PCRs were carried out in a total volume of 3 μl /well in the 384-well PCR plate. The PCR conditions were as follows: 50 °C for 2 min for the contamination control with AmpErase uracil *N*-glycosylase, 95 °C for 10 min to activate the AmpliTaq Gold Enzyme, followed by 40 cycles of 92 °C for 15 s for denaturation, and 60 °C for 1 min for annealing and extension. Amplified results at the endpoint were measured using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). The genotype for each sample was identified using our Auto-Call software with human validation. The success rate for the SNP call for the listed SNPs was more than 95%. Raw genotype data were uploaded to the database server for the following statistical association study.

Statistical analysis. Statistical analyses were performed using the SPSS statistical program version 11.0. Allelic and genotypic analyses of SNPs were performed between two groups: the anti-HCV antibody and serum HCV RNA positive group (PP group, $n = 189$); and the anti-HCV antibody positive and serum HCV RNA negative group (PN group, $n = 49$). The 189 subjects who were positive for both the anti-HCV antibody and the serum HCV RNA were further divided into two groups of significantly different ALT levels. The association tests were conducted with standard positivity tables (χ^2 tests) comparing SNP genotype frequency and case-control status using 2×2 and 2×3 contingency tables. To hypothesize mode of inheritance, 2×3 tables were collapsed into a 2×2 table. Fisher's exact test was used for a 2×2 table χ^2 analysis. Differences at values of $P < 0.05$ were considered significant.

Results and discussion

The genetic variations in individuals infected with HCV that were associated with persistent viremia and different ALT levels are shown in Table 2 and Table 3, respectively. Table 2 shows the SNPs identified with a P

Table 1
The 269 SNPs derived from 103 selected genes examined

Gene symbol	IMS-JST ID	Gene symbol	IMS-JST ID	Gene symbol	IMS-JST ID	Gene symbol	IMS-JST ID
IL1A	006817	STAT5A	046163	ALDOB	005886		075389
	006815		046159	ALB	017195		075418
	006814	NFKB1	047292		049343		075422
	006816		047290	ADH1	105338		075398
	006818	GRAP2	020842		105339	BMP8	056574
IL1B	SNP000063078		020835		105343		056580
	017197		033793	ADH2	105351		022848
	SNP000000483		020828		105350	FGF1	032063
	017198		020824		105346	FGL1	009963
IL1R1	037789		020832		105345		054941
IL1R2	017401	CABIN1	020371	ADH3	105359	FGL2	003521
	009858		020369		105352	FGF6	013105
	008500		020365		013904	FGF9	070118
	017403		020388		105358	FGF10	041885
	008492		020373		053865	EGF	054195
	017410		020383		105357		017545
IL1RL1	059017		020372		105355		054188
IL1RN	006946		020377		053862		032041
	006488	IFNG	037908	ADH7	105380		017539
IL2	063967	IFNGR2	005184		105379		032036
IL2RA	069775		021997		105374	EGFR	037677
IL2RB	033737	IFNAR1	053185		105376		008465
	029519		057221	ALDH2	032839		012799
	033735		053746		000756	TGFA	026239
	015320		031526	ALDH3A1	008286	TGFB1	013663
	015316	IFNAR2	056061		008288		013664
IL3	009879		017180	ALDH3A2	008295		013665
	001488		034422		008290	TGFB3	022523
IL4	005923		017179	AP1B1	020466		022521
	054219		056065		020470		034751
	017559		009668		020454	TGFBR3	064634
IL4R	001501	IRF1	005691		033491		043849
	010464		005685		020455		050873
	010460	IFI27	010856		007897		050868
	008008	IFI30	065201		057697		050870
IL7R	065104		045873		020462	LTBP1	042424
IL8RA	SNP000063457	IFI41	013416	AP1G1	025170	LTBP2	058392
IL8RB	rs2230054	TNF	005889		065478		058386
IL10RA	031573	TNFA	SNP000002481		065475		036941
	031574		SNP000002477	AP1G2	030324		036943
	031575	TNFR2	SNP000063488	AP1S1	010716		058391
	031572		rs945439	AP2A1	012782	LTBP4	025471
	072401	TNFRSF1A	034139		008463		025467
IL10RB	034428		017229	AP2B1	012613	CD2	016580
	035069	TNFRSF1B	071579		057894	CD4	006379
IL12A	014597	TNFRSF6	050850		057892	CD5L	051956
IL12RB1	063130		050844	SULT1A1	075306		030620
	063137		050852	SULT1A2	075337		070881
IL12RB2	071682	TNFRSF9	056147		075338	CD6	000988
IL14	029923		022127	SULT1C1	075367		000990
IL15	044018	TNFSF10	012812		075369		000993
IL15RA	050227		055636		075369		018745
	050225	TNFRSF14	030589	SULT1C2	075380		055043
	050218	TNFSF6	054103		075377		018744
IL17R	012792		017346		075373	CD34	021470
	012786		017347		075381		021467
IL18R1	012880	TRAF1	010868		075376		055591
	024760		057349		075372		021465
	012878		010872	SULT2A1	075423	CD36	005702
	037770		023072		075424	CD36L1	067469
PRL	055776	MX1	019663	SULT2B1	075438	CD36L2	041117
	047942		008786		075433		041118
	010739		003618		075435	CD48	049491

Table 1 (*continued*)

Gene symbol	IMS-JST ID	Gene symbol	IMS-JST ID	Gene symbol	IMS-JST ID	Gene symbol	IMS-JST ID
ADA	055097		033209		075437	CD74	040172
	055090		005780	SULT4A1	075408	CD80	067419
	055102	CCR2	006605		075402		
	055100		006604		075392		
STAT1	066727		054147		075419		

The JSNP ID numbers selected from the JSNP database are listed. SNP data are available at http://snp.ims.u-tokyo.ac.jp/index_ja.html

Table 2
SNPs of genes identified through comparison of subjects positive for anti-HCV antibody with HCV viremia and without HCV viremia

Gene symbol	SNP ID	SNP	Model[a]	*P* value[b]	Odds (95% CI)
IL4	IMS-JST054219	C/T	CC+CT/TT	0.0320	2.50 (1.10–5.65)
IL8RB	rs2230054	C/T	CC/CT+TT	0.0350	2.10 (1.08–4.17)
IL10RA	IMS-JST031573	A/G	GG/GA+AA	0.0490	2.29 (1.01–5.26)
			GG+GA/AA	0.0310	2.20 (1.09–4.44)
	IMS-JST031574	A/G	GG/GA+AA	0.0490	2.29 (1.01–5.26)
			GG+GA/AA	0.0310	2.20 (1.09–4.44)
	IMS-JST031575	C/T	TT/CT+CC	0.0290	2.63 (1.11–6.25)
			TT+TC/CC	0.0320	2.18 (1.08–4.41)
PRL	IMS-JST055776	A/G	GG+GA/AA	0.0030	2.75 (1.40–5.40)
ADA	IMS-JST055097	A/C	CC+CA/AA	0.0400	2.86 (1.10–7.45)
	IMS-JST055102	A/G	AA+AG/GG	0.0140	3.21 (1.33–7.75)
NFKB1	IMS-JST047292	A/T	AA/AT+TT	0.0450	3.14 (1.03–9.09)
GRAP2	IMS-JST020842	C/T	CC/CT+TT	0.0440	2.14 (1.03–4.55)
CABIN1	IMS-JST020371	C/T	CC/CT+TT	0.0300	2.05 (1.09–3.85)
IFNAR2	IMS-JST056061	A/C	AA/AC+CC	0.0220	2.31 (1.15–4.55)
IFI27	IMS-JST010856	A/G	AA+AG/GG	0.0230	2.18 (1.15–4.14)
IFI41	IMS-JST013416	C/T	TT+TC/CC	0.0350	4.14 (1.15–14.92)
TNFRSF1A	IMS-JST034139	G/T	GG+GT/TT	0.0490	2.04 (1.06–3.94)
ALDOB	IMS-JST005886	C/T	TT/CT+CC	0.0070	2.88 (1.32–6.25)
AP1B1	IMS-JST020466	C/T	TT+TC/CC	0.0150	2.31 (1.21–4.41)
	IMS-JST020470	C/T	TT+TC/CC	0.0150	2.30 (1.20–4.38)
SULT2B1	IMS-JST075438	A/G	GG+GA/AA	0.0200	5.23 (1.35–20.27)
EGF	IMS-JST054195	A/C	CC+CA/AA	0.0270	2.42 (1.13–5.20)
EGFR	IMS-JST037677	A/C	AA/AC+CC	0.0200	3.30 (1.30–8.33)
TGFB1	IMS-JST013663	G/T	TT+TG/GG	0.0140	2.42 (1.23–4.75)
	IMS-JST013665	G/T	GG+GT/TT	0.0490	2.13 (1.06–4.30)
LTBP2	IMS-JST058392	A/G	GG+GA/AA	0.00067	3.83 (1.81–8.11)
CD4	IMS-JST006379	C/T	CC+CT/TT	0.0180	2.71 (1.22–6.02)

[a] The model of significant genotypes (subjects with HCV viremia/subjects without HCV viremia).
[b] Fisher's exact test.

value of less than 0.05 between the PP group and the PN group. Table 3 shows the SNPs identified with a *P* value of less than 0.05 between subjects with a high ALT level and those with a low ALT level in the PP group. In a general sense, the inheritance of an at-risk allele follows either dominant or recessive patterns, and this could be observed from our test results. A dominant allele tends to be statistically significant for both its homozygotic and heterozygotic states, whereas a recessive allele tends to be statistically significant only for its homozygotic state.

In the current analysis, we did not apply any adjustment for multiple testing, such as a Bonferroni correction, because our purpose was to identify as many candidate genes as possible. Since subjects of this study seemed genetically homogeneous, these results may not be generalizable to other populations. Thus, the results regarding the SNPs and genes listed in this study should be interpreted with caution, and it is important to further test and replicate the results in other populations, preferably with a larger sample. It should be noted that even bona fide SNPs may not indicate causative variation, but merely act as genetic markers in linkage disequilibrium with other causative variations [12]. In spite of the limitations of our study, we believed that undertaking such a population-based study would provide information on a novel aspect of the disease, and it is interesting to investigate the genes that have emerged through the investigation. Some information derived from this study might be useful to develop new

Table 3
SNPs of genes identified in subjects positive for serum HCV RNA through comparative analyses of two groups with significantly different ALT levels

Gene symbol	SNP ID	SNP	Model[a]	*P* value[b]	Odds (95% CI)
IL1B	IMS-JST017197	C/T	CC + CT/TT	0.0430	1.91 (1.03–3.57)
			CC/CT + TT	0.0310	2.31 (1.09–4.9)
	SNP000000483	C/T	TT/CT + CC	0.0270	2.43 (1.12–5.26)
			TT + CT/CC	0.0290	2.01 (1.08–3.73)
	SNP000063078	C/T	CC/CT + TT	0.0310	2.31 (1.09–4.90)
IL1RL1	IMS-JST059017	G/T	TT + TG/GG	0.0040	2.59 (1.35–5.00)
IL2RB	IMS-JST033737	A/T	AA + AT/TT	0.0190	2.08 (1.15–3.74)
IL12RB1	IMS-JST063137	A/G	AA + AG/GG	0.0370	1.90 (1.04–3.47)
IL18R1	IMS-JST037770	A/G	GG + AG/AA	0.0120	2.33 (1.23–4.39)
STAT5A	IMS-JST046163	A/G	GG/AG + AA	0.0002	ND[c]
GRAP2	IMS-JST020842	C/T	CC + CT/TT	0.0007	4.18 (1.76–9.92)
			CC/CT + TT	0.0350	1.96 (1.07–3.59)
	IMS-JST020835	A/G	AA/AG + GG	0.0290	1.93 (1.08–3.45)
CABIN1	IMS-JST020383	A/G	GG/AG + AA	0.0094	2.46 (1.26–4.78)
	IMS-JST020372	C/G	GG/CG + CC	0.0060	2.52 (1.30–4.90)
	IMS-JST020377	A/G	GG/AG + AA	0.0098	2.48 (1.28–4.83)
IFNAR1	IMS-JST031526	C/G	GG + GC/CC	0.0200	2.14 (1.14–4.00)
MX1	IMS-JST019663	C/G	CC/CG + GG	0.0012	2.88 (1.51–5.47)
	IMS-JST008786	A/G	AA/AG + GG	0.0005	3.35 (1.71–6.55)
	IMS-JST033209	A/C	AA/AC + CC	0.0003	3.28 (1.74–6.19)
BMP8	IMS-JST056574	C/T	CC/CT + TT	0.0005	4.70 (1.91–11.6)
	IMS-JST056580	A/G	AA/AG + GG	0.0005	4.75 (1.93–11.73)
	IMS-JST022848	G/T	TT/GT + GG	0.0006	4.31 (1.81–10.31)
FGL1	IMS-JST009963	A/G	GG/AG + AA	0.0420	2.59 (1.08–6.21)
LTBP2	IMS-JST058392	A/G	AA + AG/GG	0.0070	2.37 (1.30–4.32)
	IMS-JST058391	A/T	AA/AT + TT	0.0440	2.01 (1.03–3.94)
CD34	IMS-JST021467	A/G	AA + AG/GG	0.0400	4.24 (1.13–15.95)
	IMS-JST055591	C/T	TT + CT/CC	0.0340	2.16 (1.06–4.41)
	IMS-JST021465	C/T	CC + CT/TT	0.0340	3.46 (1.06–11.31)
CD80	IMS-JST067419	A/G	AA + AG/GG	0.0006	2.93 (1.60–5.35)

The mean ALT level for the two groups was significantly different (63.7 ± 30.4 IU/L vs. 20.8 ± 4.2 IU/L; $P < 0.001$).
[a] The model of significant genotypes (subjects with the higher ALT level/subjects with the lower ALT level).
[b] Fisher's exact test.
[c] ND, not determined. No subjects with the lower ALT level showed the genotype GG.

hypotheses for future research on hepatitis C. Some of the highlights for the attractive roles of the genes are described below.

The genes listed in Table 2, which include IL4, IL8RB, IL10RA, PRL, ADA, NFKB1, GRAP2, CABIN1, IFNAR2, IFI27, IFI41, TNFRSF1A, ALDOB, AP1B1, SULT2B1, EGF, EGFR, TGFB1, LTBP2, and CD4, may reflect the genetic variations in HCV-infected individuals that are specific to susceptibility to infection, persistent viral replication, and immunity against HCV. SULT2B1 is a sulfotransferase enzyme that regulates the synthesis of heparan sulfate proteoglycan [13]. The initial event in the etiology of flavivirus infection is known to be the binding of the virion to the sulfate proteoglycan expressed on the cell surface [14,15]. Since HCV belongs to the family of flaviviruses, the same mechanism of infection might exist [16]. Genetic variations of a gene encoding sulfotransferase may reflect differences among individuals in the degree of expression of virion-binding proteins such as heparan sulfate proteoglycan on the cell surface, which may influence susceptibility to viral entry into liver cells. In the same line, SNPs of the adaptor protein coding gene, AP1B1, were listed. AP1B1 is a group of proteins associated with clathrin-coated vesicles, and these proteins interact with the cytoplasmic domains of receptors found in the plasma membrane [17,18]. Since flaviviruses use the major clathrin-dependent endocytic pathway during infection [15], SNPs of AP1B1 may be associated with the genetic differences that influence the entry of virions into the liver cell. Genetic variations of the above two genes in individuals may influence their susceptibility to viral attachment to liver cells, which is essential for the establishment of infection and persistent re-infection in chronic carriers of HCV. The identity of the receptor for HCV in the liver cell membrane is still obscure [19–21], but the differences between chronic HCV carriers and those without HCV viremia in genes encoding proteins that bind virions before attachment of HCV to the receptor suggest a novel future strategy for control of HCV infection. SNPs of genes that may affect T cell immunity in infection, such as GRAP2 and ADA, were also listed. GRAP2 is an immune cell-specific adaptor protein specifically expressed in lymphoid tissues to

activate T lymphocytes [22]. ADA forms a complex with the cell membrane protein CD26/dipeptidyl-peptidase IV that has an essential role in immune regulation as a T cell activation molecule, and CD26-bound ADA has been postulated to regulate extracellular adenosine levels and to modulate the costimulatory function of CD26 on T cells [23]. Such T cell regulation may be important in controlling immune responses to HCV.

The genes listed in Table 3, which include IL1B, IL1RL1, IL2RB, IL12RB1, IL18R1, STAT5A, GRAP2, CABIN1, IFNAR1, Mx1, BMP8, FGL1, LTBP2, CD34, and CD80, are most likely to be associated with the genetic variations affecting cell cytotoxicity based on immune responses against HCV in viral carriers. The SNPs of Mx1, CD80, BMP8, and STAT5A showed low P values (less than 0.001), and thus the SNPs of these genes are more likely to have a true association with liver necro-inflammation, probably through active cytotoxic immune responses in HCV infection. This study is the first to demonstrate SNPs of the human Mx1 gene in HCV carriers through comparison of two groups with significantly different ALT levels. Mx1 protein may be a key factor in inhibiting the multiplication of HCV as well as other single-strand RNA viruses in the host [24]. Recent reports have shown that SNPs in the Mx1 gene promoter are correlated with the response of hepatitis C patients to interferon therapy [25,26]. Even in the natural course of HCV infection, the genetic variations represented by SNPs in the Mx1 gene may influence the Mx1 protein induction by anti-viral cytokine responses during viral infection and lead to differences in the outcome of HCV infection. Engagement of costimulatory molecules such as CD28 or CTLA4 on T cell surfaces by CD80 (B-lymphocyte activation antigen B7-1) provides a costimulatory signal to T cells for IL2 production and cell proliferation [27]. Thus, the SNPs of genes coding CD80 may represent genetic variations that influence an interaction between T and B cells for activation of an immune response in HCV infection. BMP8 is a member of the TGF-β superfamily [28]. Natural killer cells are known to produce large amounts of TGF-β, which have an inhibitory effect on lymphocytes [29]. As TGF-β has an important role in the regulation of T cells, the genetic diversities represented by SNPs in both TGF-β and its superfamily genes may influence the outcome of HCV infection. The principal IL2-inducible component bears significant relatedness to a PRL-induced transcription factor, STATs. Activation of human STAT5 is believed to govern the biological effects of IL-2 during the immune response [30,31]. The SNPs of genes coding STAT5, PRL, interleukins, and their receptors shown in this study may also represent important genetic variations that influence the host's immune responses with HCV infection. Interestingly, multiple SNP sites of the gene encoding CABIN1 were identified in this study. Investigation of the function of CABIN1 in immunity against HCV is an ideal topic for future research.

In conclusion, we have identified some genetic variations associated with differences in the course of HCV infection based on a population study. The SNPs identified in HCV-infected humans in this study are worth confirming in another larger cohort. It is important to define the function of these genes with regard to HCV infection and host defense mechanisms.

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Transmission of hepatitis C virus quasispecies between human adults

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Abstract

To elucidate how hepatitis C virus (HCV) with multiple variants (quasispecies) is transmitted and adapts to the host during infection, we compared nucleotide and deduced amino acid sequences from hypervariable region1 (HVR1) of the E2 gene of HCV between a donor and a recipient who developed hepatitis after a needlestick accident. Thirty clones from each subject were sequenced after PCR amplification, cloning, and purification of plasmid DNA from single colonies of transformed bacteria. Genetic analysis revealed that the recipient's viral sequences were much less diverse than the donor's. We found a single predominant HCV HVR1 clone of the recipient in 22/30 isolates with the same amino acid sequence, and mimic clones in 8/30 isolates with only one amino acid substitution. These were all absent in the donor, who had 21 highly diverse sequences. Phylogenetic analysis of virus E1/E2 gene sequences showed that the recipient's unique sequences were related to the population of variants from the donor, in whom one isolate had 96% similarity to the recipient's predominant amino acid sequence. These results suggest that a minor subset of the donor's HCV variants is selectively transmitted to the recipient, and that the selection determines the predominant variant in the new host.
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Keywords: HCV; Envelope protein; Hypervariable region; Variant; Needlestick accident; Interferon

1. Introduction

Genetic analysis of the hepatitis C virus (HCV) has revealed that HCV has significant genetic heterogeneity among isolates, and that the degree of variability is unevenly distributed throughout the viral genome: some regions are conserved and some are highly variable [1]. The HCV genome, especially the hypervariable region1 (HVR1) of the E2 gene, which encodes a putative envelope glycoprotein, mutates at a high rate resulting in a large spectrum of mutants referred to as quasispecies during infection [2,3]. Some virions may contain defective RNA genomes, which also affect the infectivity and replicability of the virus [4]. The mixture of clones determines the biological and immunological properties of the virus.

How HCV with multiple variants in the infected human is transmitted and subsequently causes hepatitis in the recipient is not well understood. Which HCV variants are transmitted remain unknown. The horizontal transmission of HCV through needlestick accidents is a serious problem among health workers [5] because an effective HCV vaccine is not available. We experienced a case in which HCV transmission occurred from an HCV-infected patient (donor) to a nurse (recipient) through a needlestick accident in the hospital. The recipient developed hepatitis 6 weeks later. The donor's serum was frozen at the time of the accident, and the recipient's one was also periodically collected after the onset of the accident. Thus, we were able to analyze the transmission mode of the viral population between the two. The transmission mode of HCV variants between humans has not been clarified. To

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elucidate how HCV quasispecies are transmitted between human adults, we analyzed multiple sequences of the most variable region of the HCV genome, HVR1. The HVR1 is believed to contain important B-cell epitopes for virus neutralization, and this variability may provide an immune escape mechanism allowing viral persistence in the host [6]. Our results give a better understanding of how HCV infection occurred and how multiple variants adapted to the recipient. These findings are important in considering both therapy and prevention of hepatitis C.

2. Materials and methods

2.1. Virological assays

Antibodies to HCV (anti-HCV) and serum HCV RNA were examined by third-generation enzyme-linked immunosorbent assay (Ortho Clinical Diagnostics Inc., Raritan, NJ) and the Amplicor HCV RNA detection kit (Nippon Roche, Tokyo, Japan), respectively. HCV typing was carried out by a PCR-based genotyping assay using type-specific primers [7].

2.2. Donor and recipient

The donor was a 65-year-old woman suffering from gastric cancer. She had chronic HCV infection positive for both anti-HCV and HCV RNA. The HCV genotype of the donor was 1b (HCV-1b). The recipient was a 35-year-old nurse who had no evidence of hepatitis for at least seven years before the accident, according to the records of a health examination program for health workers. She accidentally dropped a 16-gauge needle, which pierced her left foot, after taking blood from the donor patient. She washed her foot immediately and then reported the accident to the hospital. The donor's serum was collected and frozen immediately after the accident, and the recipient's one was also periodically collected after the onset of the accident.

2.3. Cloning and sequencing of the HCV envelope region

For cloning and sequencing of the HCV HVR1, HCV RNA was extracted from 100 ml of serum using the Sepa Gene-RV kit (Sanko Chemical Co., Tokyo, Japan). The complementary DNA (cDNA) was synthesized using 200-u Moloney murine leukemia virus reverse transcriptase with a 100-pmol random primer. The cDNA was then amplified by nested PCR using primers derived from the envelope region based on the published nucleotide sequences of HCV-J [8] that belongs to the HCV-1b. Nucleotide positions (nt) are numbered according to the HCV-J sequence [8]. Primers were designated for the 301-bp amplification product (nt 1310–1610) covering part of the HCV E1 and E2 regions containing HVR1 (nt 1479–1559): the outer primers for the first-stage PCR were 5′-GCC ACG TAT CAG GTC ACC GCA TGG C-3′ (nt 1261–1285) and 5′-GCT CCG GGC ACC CGG ACG AGT TGA A-3′ (nt 1668–1692). The inner primers for the second-stage PCR were 5′-GCT TGG GAT ATG ATG ATG AAC TGG TC-3′ (nt 1284–1309) and 5′-GGT GTG GAG GGA GTC ATT GCA GTT-3′ (nt 1611–1634). Molecular cloning was done with an Original TA cloning kit (Invitrogen Co., Carlsbad, CA). The PCR product was ligated into the pCR 2.1 vector and transformed into competent cells. Plasmid DNA was then amplified in *Escherichia coli* and purified with a Qiagen purification kit (Qiagen, Inc., Chatsworth, CA). Thirty clones from each subject were analyzed. The clones were lettered D for donor or R for recipient, and numbered from 1 to 30. The insert DNA was sequenced using the 5′ sequencing primer (M13 reverse primer) 5′-CAG GAA ACA GCT ATG AC-3′ and the 3′ sequencing primer (M13 forward primer) 5′-GTA AAA CGA CGG CCA G-3′ with the fluorescent dye terminator cycle method in an ABI 310 automated sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences of these isolates were then aligned with other reported sequences of HCV-1b. To establish the relatedness of these viruses, part of E1 genome region (nt 1311–1478) as well as HVR1 was analyzed. The evolutionary relationship of virus E1/E2 gene sequences (nt 1311–1610) between these samples and previously reported isolates was elucidated by the six-parameter method [9]. A phylogenetic tree was constructed by the unweighted pair-group method with arithmetic mean using the ODEN program [10].

3. Results

3.1. Case report

Fig. 1 shows the case profile of the needlestick accident and the clinical characteristics of the recipient. Three days after the accident, the nurse was tested for her blood biochemistry values and anti-HCV; her biochemistry values were normal, and anti-HCV was negative. Six weeks after the accident, the recipient developed acute hepatitis. She complained of general fatigue and had a high alanine aminotransferase level of 646 IU/L (normal < 30 IU/L). Anti-HCV seroconverted to positive at this point, and the recipient's serum sample was collected and frozen. The cut-off index of anti-HCV gradually increased after seroconversion. The genotype of the recipient was HCV-1b, and the viral amount determined by the Amplicor HCV moniter assay (Nippon Roche) was 840 K copies per ml. Liver biopsy 20 days after onset of hepatitis showed severe necroinflammatory changes of the liver. The recipient developed symptoms of chronic hepatitis, because the serum HCV RNA became positive with a fluctuation of ALT levels. She took interferon (IFN) therapy for 24 weeks using the beta IFN with a total administration of 576 megaunits. This therapy was effective and brought the complete response sustaining the eradication of HCV RNA and normal liver function tests after termination of IFN administration.

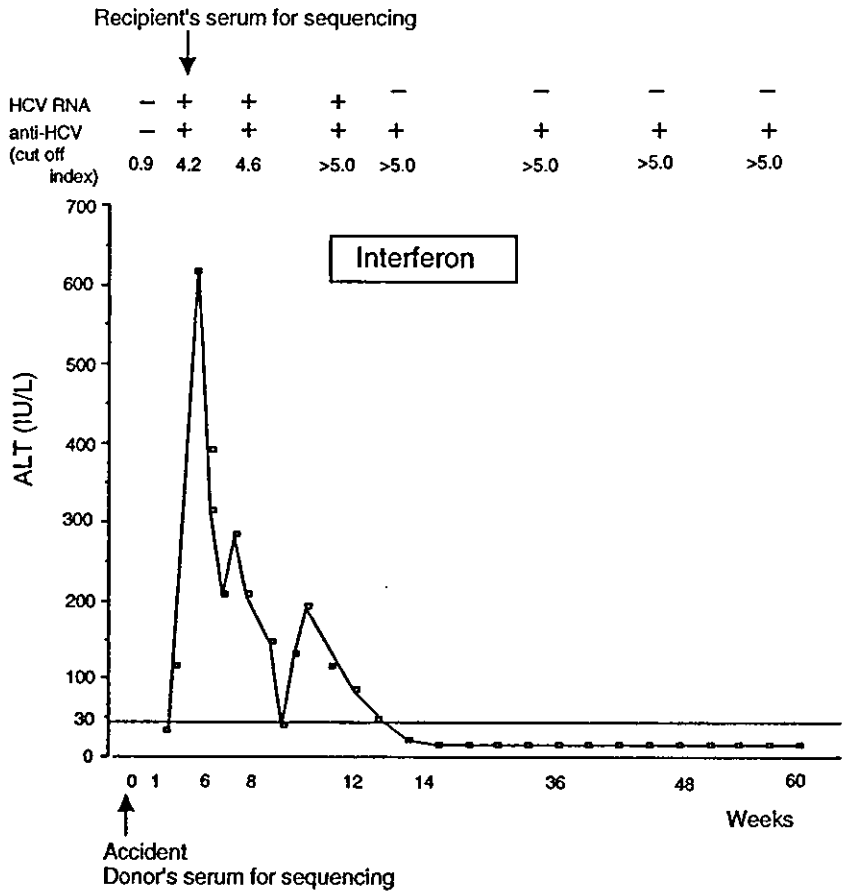

Fig. 1. Clinical course of the recipient. Arrows denote sampling points at which sequences of HCV HVR1 were determined.

3.2. *Transmission of HCV quasispecies between human adults*

Figs. 2 and 3 show the nucleotide and deduced amino acid sequences of 30 individually subcloned HVR1 cDNAs from each subject. Nucleotide sequences of 20 recipient clones (represented as consensus [R01] in Fig. 2) were the same; the other 10 clones had almost the same sequences as the major isolate, showing only one nucleotide substitution. The nucleotide change in 2 of these 10 clones was a silent mutation, not accompanied by a change of deduced amino acid. Thus, the deduced amino acid sequences of 22 isolated recipient clones were the same, and 8 clones were very similar. The recipient was not a hypogammaglobulinemic patient, in whom genetic variation of a virus is reduced through the lack of humoral immune selection pressure [11]. The nucleotide and deduced amino acid sequences of donor clones had a large spectrum of sequences, showing quasispecies distribution. Nucleotide sequences of 26 donor clones were diverse and had a large number of substitutions. There were 21 deduced amino acid sequences. Four isolates, represented by D05 in Fig. 3, were the major HCV HVR1 sequences in the donor, but were not detected in the recipient. No identical nucleotide and amino acid sequences of HCV HVR1 were found between donor and recipient. However, when we analyzed the deduced amino acid sequences of 30 individually subcloned part of HCV E1 cDNAs from each subject, we found that amino acid sequences of 16 recipient clones were identical with those of 15 donor clones with the same sequence. A phylogenetic tree constructed from the 300-bp cPCR nucleotide sequences covering part of E1 and E2, containing HVR1, showed that the nucleotide sequences found between donor and recipient were related and derived from the same cluster (Fig. 4). The closest sequences were between R01, the predominant variant in the recipient, and D18, which belonged to a minor subset in the donor. These had 96.3% similarity in nucleotide sequences and 96.0% similarity in deduced amino acid sequences, when compared these two 300-bp cPCR products.

4. Discussion

The results of this study suggest that HCV variants are transmitted selectively, and that the selection determines the predominant variant in the new host, which might have been a minor variant in the former host.

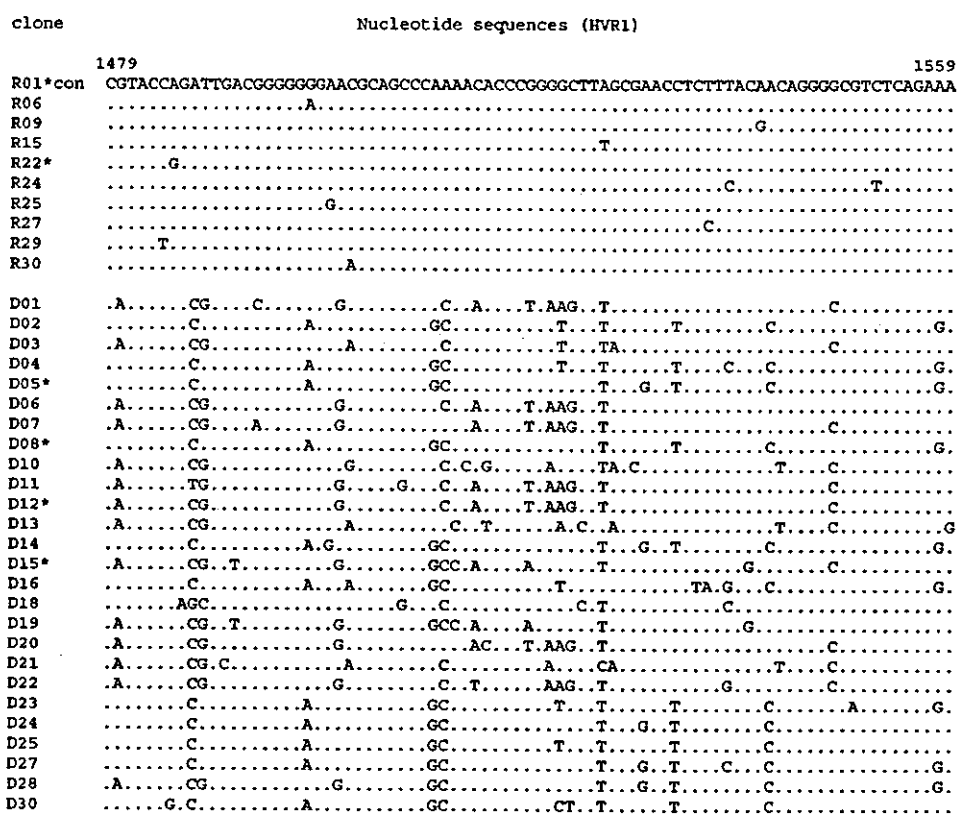

Fig. 2. Nucleotide sequences of subcloned cDNA products from the HCV HVR1 derived from recipient (R01–R30) and donor (D01–D30). Points represent nucleotides identical to the consensus sequence (con), given on the top line, for the major viral species found in the recipient (R01). (*) The nucleotide sequences of clones R02–R05, R07, R08, R10–R14, R16–R21, R26, and R28 are identical with the sequence of R01; R23 with R22; D09 with D05; D26 with D08; D17 with D12; and D29 with D15.

Studies of the transmission of HCV quasispecies have been done in chimpanzees — the only reliable model of HCV infection. A comparison of amino acid sequences of HCV HVR1 clones between human serum-derived inocula and infected chimpanzee sera showed that the major variant in the inocula was transmitted and replicated in the chimpanzee [12]. However, in another chimpanzee experiment using the same inocula, the same major variant was not recovered, but a minor variant, which was not recovered in the first experiment, was recovered [13]. These results suggest that both variants were infectious to chimpanzees but that selection of a specific HCV clone during infection differed between the two chimpanzees. The infectivity of HCV clones has also been found to be individually different between human and chimpanzee [14]. Why a particular clone from a heterogeneous mixture of variants is transmitted to the recipient and replicates as the major clone in the new host has not been elucidated. Several studies have revealed that a single HCV clone can be infectious and have isolated such infectious sequences in HCV genotype 1a and 1b [15–17]. The variant that becomes predominant in the new host may originate from the infectious variants with a high infectivity titer [18] or those escaping immune surveillance in the acute phase of infection in the new host [12,19]. Selective transmission of HCV variants between humans has been found in vertical transmission from mother to infant. The recipient infant also had a major predominant variant that was shown to be related to the mother's variants by phylogenetic analysis but not identical to any [20].

On the basis of the replicative characteristics of RNA viruses, mutant sequence variations of HCV are considered to range widely, and most circulating HCV virions are postulated to contain defective genomes [4]. We cannot estimate how many infectious clones are contaminated in such numerous variants in the donor blood. The existence of a single donor's clone closely related to the predominat recipient's clone may suggest that the infectious and replicable HCV variant might belong to a minor subset of variants in the donor. If so, antibodies against most of variants except for the infectious one, in which one neutralizing epitope probably exist in HVR1 [21], are not necessary to prevent infection in the initial phase. This point should be considered in a strategy for future vaccine development.

This selectivity also highlights an important clinical manifestation. Most patients with HCV infection develop chronic hepatitis with a risk of progression to cirrhosis or hepatocellular carcinoma [22]. The evolutionary dynamics of the HCV quasispecies during the acute phase of HCV infection predict whether the infection will resolve or become chronic [23]. Although we could not examine the HCV quasispecies