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肝炎等克服緊急対策研究事業

データマイニング手法を用いた効果的なC型肝炎治療法に関する研究

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厚生科学研究費補助金肝炎等克服緊急対策研究事業（肝炎分野）

データマイニング手法を用いた効果的なC型肝炎治療法に関する研究

C型慢性肝炎からの発癌予測アルゴリズム、およびペグインターフェロン・リバビリン併用療法における再燃予測アルゴリズムの構築

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研究要旨:C 型慢性肝炎においては長期経過の中で肝発癌にいたることが最も予後規定因子となる。個々の症例での肝発癌リスクを予測することは、適切な肝細胞癌サーベイランス計画の立案や、発癌抑止を視野にいれたインターフェロン治療適応判断の重要な情報となる。データマイニング手法を用いて、5 年以内に発癌するリスクを予測するアルゴリズムを構築した。武蔵野赤十字病院で IFN 治療を受けて非著効であった症例をモデル作成のために使用し、班員の施設で経過観察している症例で外部検証を行った。構築された発癌予測モデルでは、血小板数、アルブミン値、AST 値、GGT 値が発癌関連因子として抽出された。このモデルは外部検証でも再現性は良好であった。C 型慢性肝炎は肝発癌リスクが高い疾患であり、肝発癌率と治療効果の両面からインフォームドコンセントを行うことが必要となる。個々の症例で、治療しなかった場合の肝発癌率を予測することは、患者が治療を受ける機会を逸しないために重要であり、患者の同意を得るために重要な根拠になる。また、ペグインターフェロン・リバビリン併用療法では、治療中に血中から消失したHCVが治療終了後に再出現し、肝炎が再燃する症例が約 30%存在する。これらの患者は治療に反応しているため、再燃の要因を分析し、対策を講じることで治癒率を向上できる。治療開始後 12 週以内に HCV が消失した症例を対象とし、治療終了後の再燃を予測するアルゴリズムを構築した。当班の症例でモデルを作成し、八橋班と連携することで外部検証を行った。その結果、再燃と関連したのは、HCV陰性化時期、年齢と総リバビリン投与量であった。この結果は、外部検証でも再現された。本解析により、インターフェロン治療に反応し HCV が陰性化した症例からの再燃を抑止するために必要な総リバビリン量を具体的な目標投与量として示せた。

A. 研究目的

C 型肝炎ウイルス(HCV) 持続感染は 肝癌の主要な原因であるため、治療により HCV 排除を行うことにより、肝癌による死亡の大幅な減少が期待される。現在の標準治療であるペグインターフェロン・リバビリン併用療法では、ゲノタイプ 1b、高 HCVRNA 量の難治例におけるウイルス排除率は 50%にとどまる。全体のウイルス排除率を向上させるためには、治療抵抗性の要因を分析し、個々の症例における難治要因に基づいた個別化治療法を確立する必要がある。現在の標準治療よりも強力な抗ウイルス作用を有する薬剤の承認が期待されるが、発癌リスクの高い症例では、現時点での最良の抗ウイルス治療を早急に行うことで、発癌の抑止を図らなければならない。

本年度の研究では、C 型慢性肝炎において、抗ウイルス治療を行わなかった場合に発癌リスクを解析し、早急に治療が必要な発癌リスクの高い症例の囲い込み集団について検討した。またペグインターフェロン・リバビリン併用療法において、HCV 遺伝子変異を含めたウイルス側要因、宿主側要因、薬剤投与量や治療期間などの治療要因を網羅的に解析し、これらを統合的に組み入れた再燃予測アルゴリズムを確立することを目的とした。

B.研究方法

武蔵野赤十字病院でインターフェロン治療が非著効となり、その後 5 年以上経過観察した 865 例を対象とした。5 年時点での発癌と関連する因子について、データマイニング解析を行った。一般臨床医の使用を前提としたため、肝生検所見は予測因子に含めなかった。このモデルに対する外部検証コホートとして、班員施設でインターフェロン治療が非著効で5年以上経過観察中の329例を用いて、再現性を検証した。

ペグインターフェロン・リバビリン併用療法の治療効果を解析するためのデータベースは、班員施設から収集したペグインターフェロン・リバビリン併用療法施行例で、12 週以内に HCV RNA が陰性化した 951 例を対象とした。収集したすべての臨床データを説明変数とし、治療終了後の再燃を目的変数として SPSS Clementine ソフトウエアを用いてデータマイニング解析を行ない、再燃を効率的に判別する説明変数を情報理論に基づき逐次的に探索し、判別アルゴリズムを構築した。

(倫理面への配慮)

ヒトの遺伝子(DNA)に係わる実験・解析は行わない。臨床データのデータベース構築においては、氏名、年齢など個人情報を連結可能匿名化する。臨床試験の目的・方法、治療の副作用、患者に関する個人情報の守秘義務、患者の権利保護等について十分な説明を行い同意を得たうえで臨床試験を遂行した(新GCPに遵守)。既に医療保険が認められている治療法においても上記に準じて同意書を得ている。本研究の遂行においては各研究施設において必要な申請を行い、各種倫理規定を遵守した。

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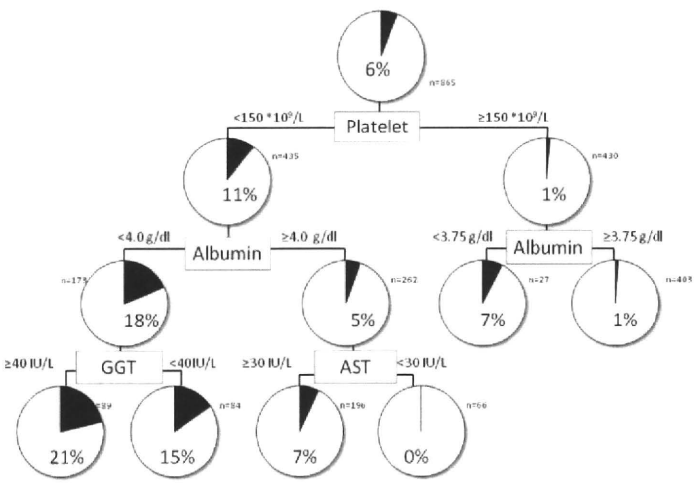
C．研究結果

発癌関連因子：武蔵野赤十字病院でインターフェロン治療が非著効となった症例で、5 年以上経過観察されている 865 例を対象として、多変量解析で発癌関連因子を検討したところ、年齢、血小板数、アルブミン値、ALT 値、γ GTP 値が独立因子であった。

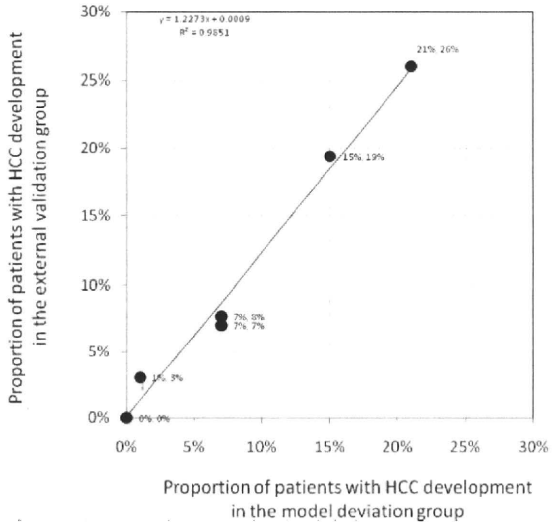
Table 2. Multivariable analysis of factors associated with HCC development within 5 years

	OR	95% C.I.	P value
Age: <60years	3.22	1.49-7.14	0.003
Alb: <4.0 g/dl	2.45	1.31-4.58	0.005
Platelet: <150*10^{9}/L	4.14	1.81-9.49	0.001
AST: >30 IU/L	9.09	1.23-1000	0.030
GGT: >40 IU/L	1.89	1.02-3.44	0.042

データマイニング解析では、血小板数、アルブミン値、ALT 値、γ GTP 値が発癌予測因子として抽出され、これらの因子の組み合わせにより、5 年発癌率が 0%から 21%までの、様々な発癌リスクを有するグループが同定された。



全体で 6%の症例で 5 年以内に肝発癌がみられたが、発癌リスク予測に最も重要であったのは血小板数であり、15 万/μl 未満が 11%、15 万/μl 以上が 1%であった。血小板低下例では、アルブミン値 4.0g/dl 未満の例が 18%の発癌リスクであり、4g/dl 以上が 5%であった。アルブミン低値例ではγ GTP が 40 IU/L 以上の発癌リスクが 21%と高く、40 IU/L 未満では 15%であった。一方、血小板低下例でアルブミン値 4.0g/dl 以上の例では 5 年間の肝発癌率は 5%であり、この中では血清 AST 値 30 IU/L 以上の群が 7%、30 IU/L 未満では 0%であった。血小板が 15 万/μl 以下の例ではアルブミン値が 3.75g/dl 未満例で 5 年発癌率が 7%、3.75g/dl 以上では 5 年発癌率は 1%であった。この解析により、発癌リスクが最も高いのは①血小板数 15 万/μl 未満、アルブミン値 4.0g/dl 未満、γ GTP40 IU/L 以上の症例で、続いて②血小板数 15 万/μl 未満、アルブミン値 4.0g/dl 未満、γ GTP40 IU/L 未満の症例、③血小板数

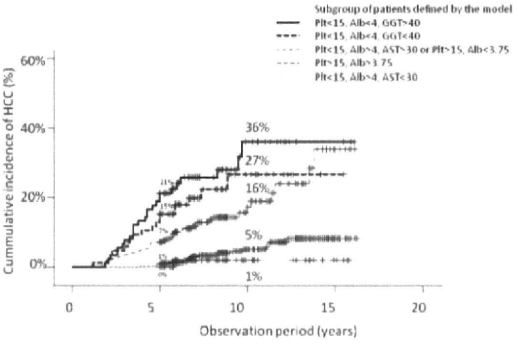


15 万/μl 未満、アルブミン値 4.0g/dl 以上、AST 値 30IU/L 以上の症例、④血小板数 15 万/μl 以上、アルブミン値 3.75g/dl 未満の症例、⑤血小板数 15 万/μl 以上、アルブミン値 3.75g/dl 以上の症例、⑥血小板数 15 万/μl 未満、アルブミン値 4.0g/dl 以上、AST 値 30IU/L 未満の症例であった。特に⑤、⑥の 2 群は、5 年発癌率が 0-1%と極めて低率であったのに対し、①、②では 15-21%と高率であり、③、④は中間のリスク 7%であった。

このような発癌リスクの層別化の再現性について、外部コホートで検証したところ、①、②の発癌率は 19-26%、⑤、⑥の発癌率は 0-3%。③、④の発癌率は 7-8%ときわめて類似しており、相関関係を解析したところ r^2=0.985 と極めて良好な相関を示した。

また、5 年発がんのリスクで層別化したグループと、5 年以降の発癌率との関連性についても確認したところ、Kaplan Meir でも、経時的な発癌率と有意に関係していることが確認された。

Figure 4. Cummulative indicence of HCC development beyond 5 years stratified by subgroups of patients defined by the model



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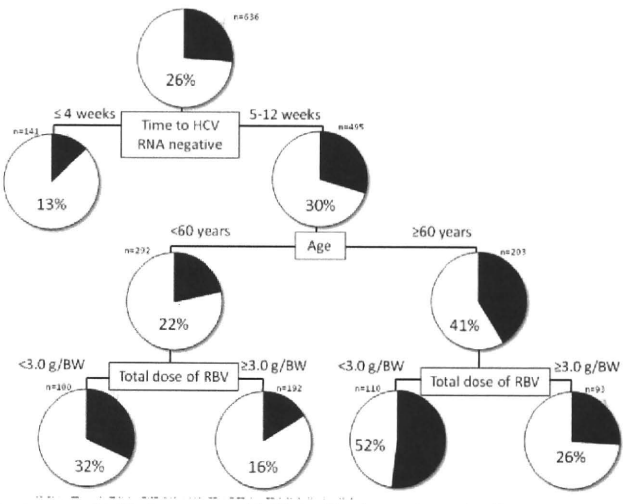
再燃の予測:12 週以内に HCVRNA が陰性化した場合(cEVR)の SVR 率は 74%と高率であった。しかしながら 26%は治療終了後に HCVRNA が再出現(再燃)した。12 週時点で HCVRNA が陰性化した場合には、再燃のリスクを考慮した治療計画を構築する必要があるために、再燃を予測するモデルを構築した。12 週以内に HCVRNA が陰性化した 951 例を対象とし、ランダムに抽出した 636 例で再燃予測モデルを作成し、残りの 315 例で内部検証を行った。多変量解析では、再燃と関連する有意因子は、年齢、クレアチニン値、HCVRNA 陰性化時期、そして総 RBV 投与量であった。

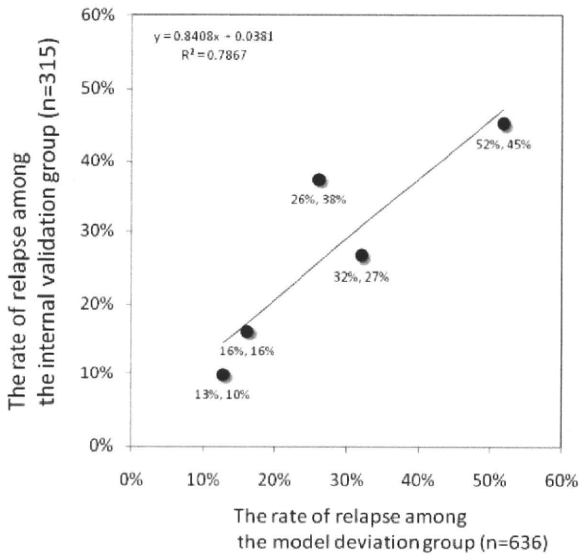
Table 3. Factors associated with relapse among patients whose HCVRNA became negative within 12 weeks after the start of therapy

	OR	95% C.I.	p value
HCVRNA negative at 4weeks	0.25	0.16-0.39	<0.0001
Total RBV dose <3g/weight(kg)	2.19	1.58-3.03	<0.0001
Creatinine <0.7g/dl	1.67	1.22-2.29	0.001
Age <60years	0.42	0.31-0.58	<0.0001

データマイニング解析では、再燃と最も関連する因子は HCVRNA 陰性化時期であり、4 週以内に HCVRNA が陰性化する RVR からの再燃率は 13%、5-12 週で HCVRNA が陰性化した cEVR からの再燃は 30%であった。cEVR の中でも、60 歳以上の高齢者では再燃率が 41%、60 歳未満では 22%であった。それぞれの群において、RBV 総投与量が 3g/kg 未満では再燃リスクが高かった。この解析により、再燃率が 13-52%での様々な再燃リスクを有するグループが同定できた。最も再燃リスクが低いのは①4 週以内に HCV RNA が陰性化した症例の 13%であり、続いて②cEVR、60 歳未満、RBV 総投与量が 3g/kg の 16%、③cEVR、60 歳以上、RBV 総投与量が 3g/kg の 26%であった。RBV 総投与量が 3g/kg 未満では再燃リスクが高く、④cEVR、60 歳未満、RBV 総投与量が 3g/kg 未満では 32%、

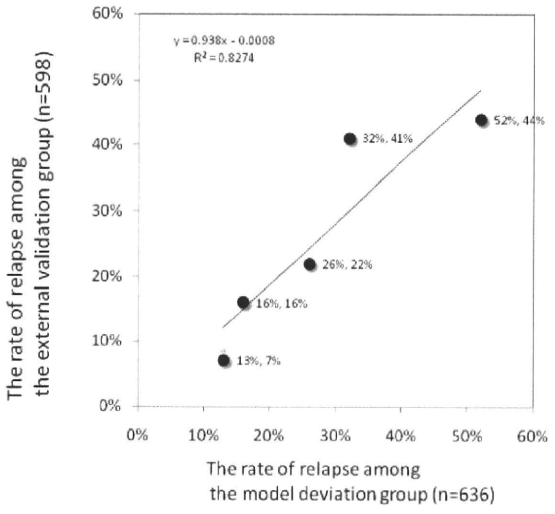


⑤cEVR、60 歳以上、RBV 総投与量が 3g/kg 未満では 52%であり、このように総 RBV 量が再燃率と密接に関連している点は注目すべきポイントと考えた。このモデルの再現性を確認するために、モデル作成には使用しなかった



内部検証コホートをあてはめたところ、再燃率は①7%、②16%、③38%、④27%、⑤45%で、相関係数は r^2=0.787 と良好な相関を認めた。

また八橋班と連携することにより、国立病院機構で収集した 598 例をあてはめて外部検証を行った。八橋班では、全体の再燃率は、21%とやや低く、モデルに当てはめた再燃率は①10%、②16%、③22%、④41%、⑤44%であり、相関係数は r^2=0.827 と良好な相関を認めた。総 RBV 量と再燃率の関連は再現性があることから、cEVR では薬剤 adherence を維持するか治療期間を延長して RBV 総投与量 3g/kg を確保することが重要であることが示された。



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D. 考察

C 型慢性肝炎において発癌リスクを予測するモデルを作成した。このモデルを使用することで、簡単な一般検査の組み合わせにより、発癌リスクを同定できる。この情報は、肝細胞癌サーベイランスの計画立案、あるいは発癌抑止を視野に入れた抗ウイルス治療の検討に際して、科学的エビデンス基いた説得力のあるインフォームドコンセントとなる。また、ペグインターフェロン・リバビリン併用療法に反応したが、治療終了後に再燃した症例を分析することで、RBV 総投与量 3g/kg 以上が治癒率を向上させるための目標薬剤投与量であることを確認した。従来は 12 週以内に HCV が陰性化した症例では 48 週間の標準治療を行っていたが、目標薬剤投与量を意識することで、早期に RBV を減量した症例では治療期間を延長するなど、再燃を抑止するための具体的な個別化医療が可能になると期待される。このモデルは、八橋班との連携により精度の高い外部検証を行うことにより、再現性、一般性が確認できた。これらの情報を、一般臨床医に広く周知することにより、治療に反応しているものの SVR が得られなかった症例の治癒率を向上させられると期待する。発癌のリスクと治癒の確率を予測することは、治療の費用対効果を考慮する上でも重要である。

E. 結論

データマイニング解析により、発癌リスクを予測するモデルと、治療後の再燃を予測するモデルを作成した。C 型慢性肝炎は肝発癌リスクが高い疾患であり、肝発癌率と治療効果の両面からインフォームドコンセントを行うことが必要となる。個々の症例で、治療しなかった場合の肝発癌率を予測することは、患者が治療を受ける機会を逸しないために重要であり、患者の同意を得るために重要な根拠になる。また、ペグインターフェロン・リバビリン併用療法で、治療中に血中から消失したHCVが治療終了後に再出現する再燃の要因を分析し、再燃を抑止するために必要な総リバビリン量を具体的な目標投与量として示せた。これらのモデルを一般臨床の場で活用することにより、患者が治療選択する際に、科学的エビデンスに基いた説得力のあるインフォームドコンセントが可能となる。

F. 健康危険情報

特記すべきことなし。

G. 研究発表

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2.学会発表

(1) Prediction of Response to Pegylated-Interferon for Chronic Hepatitis C: Impact of SNPs near the IL28B gene and Mutations in the ISDR of HCV Revealed by Data Mining Analysis. AASLD（2010 年 10 月）

H. 知的財産権の出願・登録状況

今回の研究内容については特になし。

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Original Article

Accumulation of refractory factors for pegylated interferon plus ribavirin therapy in older female patients with chronic hepatitis C

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Aim: Several host and viral factors have been reported to influence the effectiveness of pegylated interferon plus ribavirin combination therapy for chronic hepatitis C. In Japan, where the age of treated patients is comparatively high, recent studies have reported poor response to treatment in older female patients, but little is known about the relationship between advanced age in women and previously reported factors.

Methods: Using a database of 1167 patients chronically infected with hepatitis C virus (HCV) genotype 1b, we analyzed the amino acid sequences of the HCV core protein and interferon sensitivity determining region (ISDR) and examined the relationships among predictive factors.

Results: The proportion of patients with substitutions at core 70, which is associated with poor response to pegylated interferon plus ribavirin therapy, increased with age only in female patients. A similar trend was observed for ISDR wild type (wt). We also found that core 70 wt is associated with core 91 wt ($P = 5.4 \times 10^{-9}$) as well as ISDR wt ($P = 0.025$). HCV RNA levels were higher in patients with core and ISDR wt ($P < 0.001$). Furthermore, core amino acid mutations were associated with advanced fibrosis and higher inflammatory activity ($P = 0.028$ and 0.048, respectively) as well as higher gamma-glutamyltranspeptidase, alanine aminotransferase and low-density lipoprotein cholesterol levels ($P < 0.001$, 0.006 and 0.001, respectively).

Conclusion: A combination of factors account for poor response rate in older female patients in Japan. Elucidating the relationship between amino acid substitutions and metabolic alteration is an important step in understanding the mechanism of HCV interferon resistance.

Key words: combination therapy, core protein, genotype 1b, interferon sensitivity determining region, low-density lipoprotein cholesterol

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INTRODUCTION

HEPATITIS C VIRUS (HCV) is a causative agent of acute and chronic hepatitis as well as liver cirrhosis and hepatocellular carcinoma.[1–3] The single stranded RNA genome encodes one large open reading frame that is processed into at least 10 proteins by host and viral enzymes.[4,5] Some viral proteins are known to affect the outcome of pegylated interferon (PEG IFN) plus ribavirin combination therapy, the current standard of care for chronic hepatitis.[6–8] The number of amino acid substitutions in the IFN sensitivity determining region (ISDR) of the NS5A protein, which was initially reported to affect IFN monotherapy,[9,10] has recently been reported to affect PEG IFN plus ribavirin combination therapy as well.[11–14]

NS5A PKR binding domain (PKRBD),[15–19] variable region 3 (V3),[20–23] IFN/ribavirin resistance determining region (IRRDR),[24,25] and E2 PKR-eIF2α phosphorylation homology domain (PePHD)[26] have also been reported to affect therapy outcome, although these results need to be confirmed. More recently, amino acid (a.a.) substitutions in the core protein have been reported to negatively affect IFN plus ribavirin therapy.[27,28] Substitution at a.a. 70 of the core protein (core 70) has been reported to be associated with non-virological response (NVR), and this finding was confirmed by several groups.[29–31]

Several cytokines and adipokines have also been reported to be associated with the effectiveness of therapy. For instance, tumor necrosis factor (TNF)-α expression has been reported to be elevated in patients with HCV infection, and high expression levels are associated with poor response to IFN therapy.[32] IP-10 has also been reported to associate with response to therapy in patients with HCV and HIV co-infection.[33] Leptin and adiponectin levels are also reportedly associated with the effect of combination therapy.[34,35] In addition to these factors, there are many studies reporting relationships between common polymorphisms in the human genome and outcome of IFN therapy.[36–44] Among them, single nucleotide polymorphisms (SNP) in the interleukin (IL)-28B locus discovered through genome-wide association studies appear to have a large effect on outcome of PEG IFN plus ribavirin combination therapy[42–44] as well as spontaneous eradication of HCV.[45]

In addition to the above viral and host genetic factors, several metabolic factors such as obesity,[34] insulin resistance[46] and low-density lipoprotein (LDL) cholesterol levels[28,47] have been reported to be correlated with the effect of combination therapy. Furthermore, higher gamma-glutamyltranspeptidase (γ-GTP) levels, often associated with fatty liver, have also been reported to be associated with treatment outcome.[48,49] Although these factors may be mutually interdependent, their relationships with viral factors have not yet been analyzed.

Recent papers have reported poor response to therapy in older female patients,[50–52] but little is known about the relationship between age, sex and other predictive factors. To analyze these associations, we constructed a database consisting of 1425 patients with chronic hepatitis C. Using this database, we analyzed the relationship between viral and metabolic data and found that a.a. substitutions in the core and ISDR are associated with metabolic change, which may be related to disease progression and response to therapy.

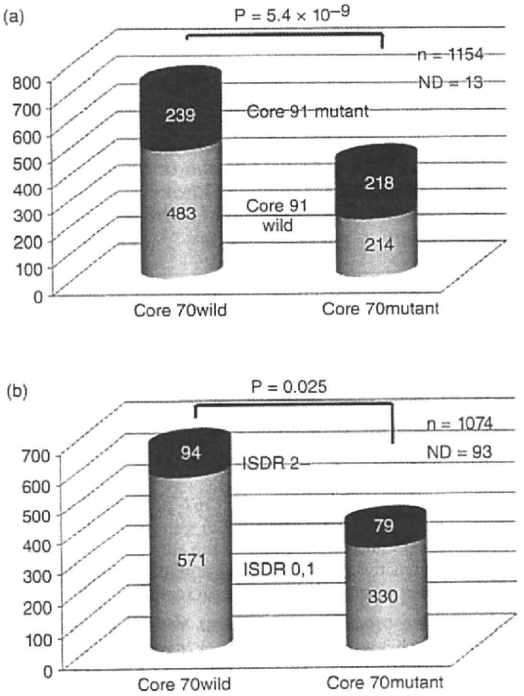


Figure 1 Association of core amino acid 70, amino acid 91 and interferon sensitivity determining region (ISDR). The relationship between hepatitis C virus core 70 and core 91 wild type and mutant amino acids (a) and the ISDR (b) were examined. Statistical significance was assessed using the χ^2-test. ND, not determined due to polymerase chain reaction or sequence calling failure.

Table 1 Clinical profile of 1167 patients

	All patients $n = 1167$	Tx naive $n = 570$ (48.84%)	Prev. tx $n = 597$ (51.16%)	*P*-value
Sex (male/female)	606/561	259/311	347/250	1.45E-05
Age	55.1 ± 10.7	55.2 ± 11.0	55.0 ± 10.5	0.604
Body weight	60.6 ± 10.8	59.5 ± 10.5	61.7 ± 11.0	0.001
BMI	27.0 ± 7.38	24.3 ± 5.46	29.6 ± 8.02	0
Fibrosis stage (0–2/3–4/ND)	815/192/160	422/78/70	393/114/90	0.005
Activity stage (0–1/2–3/ND)	531/465/171	263/234/73	268/231/98	0.803
Steatosis (present/absent/ND)	207/428/532	103/175/292	104/253/240	0.034
White blood cells (/mm^3)	4808 ± 1428	4871 ± 1395	4748 ± 1457	0.127
Hemoglobin (g/dL)	14.1 ± 1.88	14.0 ± 1.39	14.3 ± 2.23	0.001
Platelets (×10^4/mm^3)	16.6 ± 5.06	16.5 ± 5.31	16.7 ± 4.82	0.288
ALT (IU/L)	66 ± 52	67 ± 48	65 ± 55	0.265
AST (IU/L)	65 ± 54	58 ± 37	71 ± 66	0.001
γ-GTP (IU/L)	56 ± 58	57 ± 62	55 ± 54	0.942
Albumin (g/dL)	4.00 ± 0.375	4.04 ± 0.402	3.97 ± 0.347	0.001
Total cholesterol (mg/dL)	173 ± 32.1	175 ± 32.7	172 ± 31.6	0.206
Fasting blood sugar (mg/dL)	101 ± 24.9	102 ± 27.2	99.8 ± 22.2	0.715
HCV RNA (KIU/mL: amp)	2999 ± 4523	2822 ± 4365	3169 ± 4668	0.048
ISDR (0–1/≥2/ND)	908/178/81	440/85/45	468/93/36	0.863
Core 70 (wild/mutant/ND)	722/433/12	349/218/3	373/215/9	0.509
Core 91 (wild/mutant/ND)	697/457/13	349/217/4	348/240/9	0.39

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; ND, not determined; tx., treatment.

METHODS

Study subjects

WE COLLECTED DATA from 1425 participating patients with chronic hepatitis C from 16 centers in Japan. Inclusion criteria included testing positive for HCV RNA over a period of more than 6 months and testing negative for both hepatitis B virus surface antigen and anti-HIV antibody. Patients with confounding liver conditions were excluded, as well as patients who were lost to follow up or who did not have high viral load (≥5 log IU/mL) for HCV genotype 1b (Fig. 1). Patient data was not used when we failed to determine core 70, core 90 and ISDR sequences. In total, data from 1167 patients were included in the analysis. All subjects gave written informed consent to participate in the study according to the process approved by the ethical committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

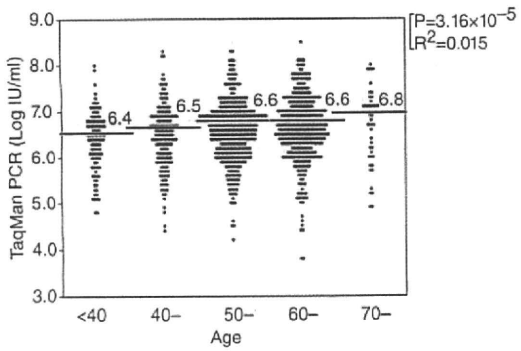


Figure 2 Relationship between age and virus titer. Virus titers were plotted according to age. The median titer within each 10-year age group is shown as horizontal bars.

Patients received weekly injections of PEG IFN-α-2b for either 48 or 72 weeks using the following doses: 60 μg for 35–45 kg bodyweight; 80 μg for 46–60 kg; 100 μg for 61–75 kg; 120 μg for 76–90 kg; and 150 μg for 91–120 kg. Ribavirin was administrated p.o., and the dose was determined based on the patient's bodyweight (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Ribavirin dosage was reduced when hemoglobin levels reduced to 10.0 g/dL and stopped if hemoglobin levels reached 8.5 g/dL. Bio-

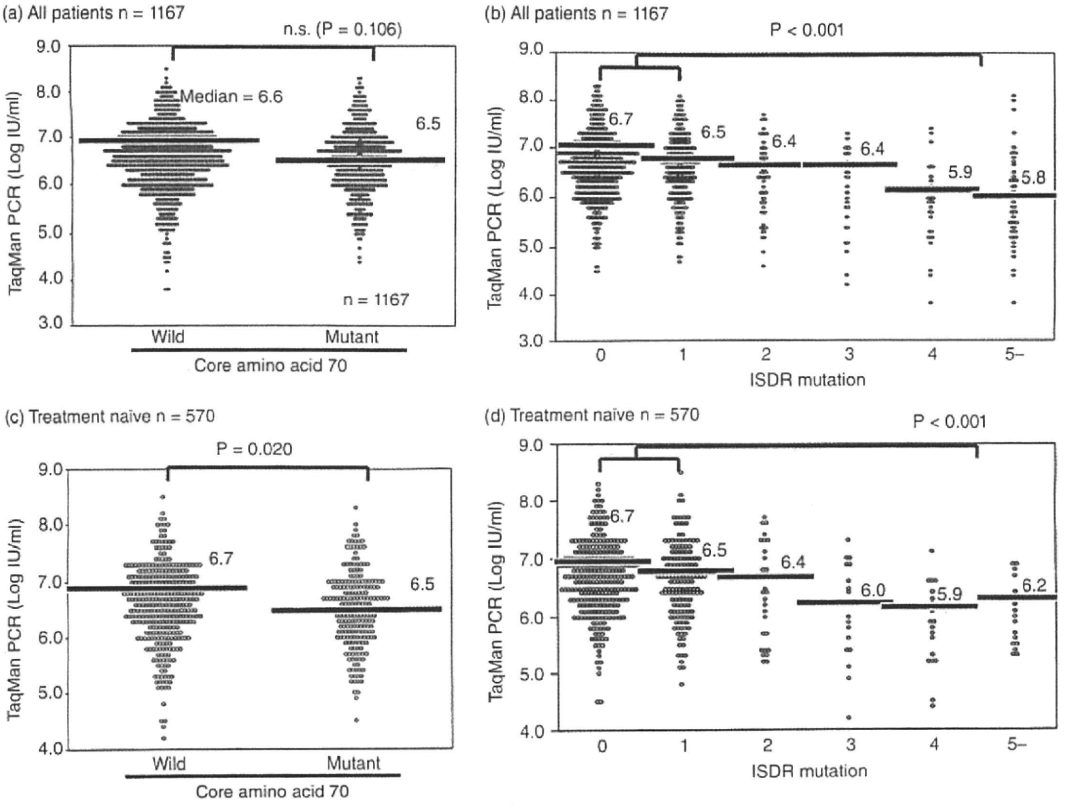


Figure 3 Analysis of virus load by core amino acid 70 substitution and number of amino acid substitutions in the interferon sensitivity determining region (ISDR). Virus titers of all 1167 patients were classified according to core 70 wild type and mutant amino acids (a) or by the number of substitutions in the ISDR (b). The 570 interferon therapy naive patients were also examined separately (c,d).

chemical tests were performed by center, and pathological diagnosis was made according to the criteria of Desmet *et al.*[53] Successful treatment was ascertained based on sustained virological response (SVR), defined as HCV RNA negative 6 months after cessation of therapy.

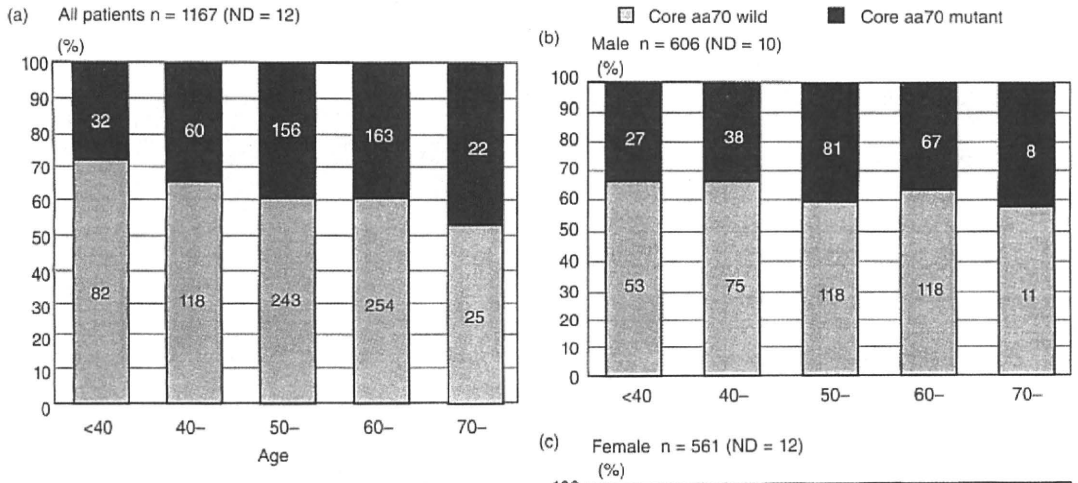
Analysis of viral titer and a.a. sequences in the core and ISDR region

The HCV RNA level was analyzed using reverse transcription polymerase chain reaction (RT–PCR)-based methods (Amplicor Hepatitis C Virus test: Roche Diagnostics, Basel, Switzerland; high range test: Cobas Amplicor, Roche Diagnostics, Basel, Switzerland; or TaqMan RT–PCR test: Applied Biosystems, Foster city, CA, USA). The measurement ranges of these assays were 5–5000 KIU/mL and 1.2–7.8 log IU, respectively. For values exceeding the measurable range, the titer was determined after dilution of the serum samples.

Sequences were determined by direct sequencing of PCR fragments following extraction and RT of serum HCV RNA. For core 70 and 91, arginine and leucine were considered wild type (wt) according to Akuta *et al.*[27,28] The number of a.a. substitutions in the ISDR was determined as described previously.[9,10,53]

Statistical analysis

The χ^2-test and Mann–Whitney *U*-test were applied to detect significant associations using PASW ver. 18



Age	Core70 Wild	Core70 Mutant
<50	200	92
≥50	522	341

P = 0.015

Age	Core70 Wild	Core70 Mutant
<50	128	65
≥50	247	156

n.s. (P = 0.234)

Age	Core70 Wild	Core70 Mutant
<50	72	27
≥50	275	185

P = 0.016

Figure 4 Age-dependent increase in core amino acid 70 mutants in female patients. Percentages of core wild type (arginine) and mutant amino acids for all patients (a), as well as for male (b) and female (c) patients are shown. Note that the age-dependent increase in mutant frequency was observed only in female patients. Statistical analysis was performed by χ^2-test. ND, not determined.

(SPSS, Chicago, IL, USA). All statistical analyses were two sided, and $P < 0.05$ was considered significant. Simple and multiple regression analyses were used to examine the association between viral substitutions and clinical factors using $P < 0.05$ as the criterion for inclusion in the multivariate model. Continuous variables were split into indicator variables based on the median, except for age which was divided into 10-year intervals. Multivariate logistic regression analysis was performed using the Design package in R (www.r-project.org) with fast backward elimination and validation based on AIC score.

RESULTS

Patient characteristics

PATIENT PROFILES ARE shown in Table 1. Results are presented separately for patients who were naive to IFN therapy and those who had had previous IFN therapy but failed to eradicate the virus.

Virus titer and a.a. substitutions in the core and the ISDR

We found a significant positive correlation between patient age and virus titer ($P = 3.16 \times 10^{-5}$, $R^2 = 0.015$,

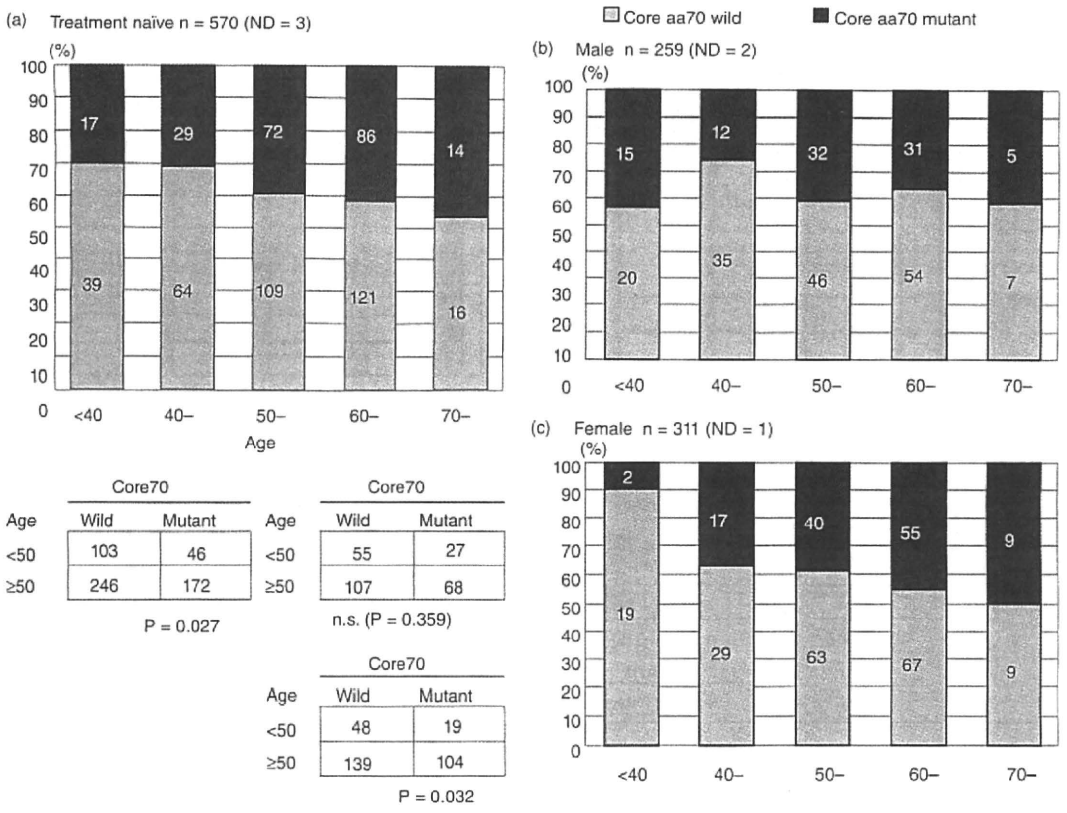


Figure 5 Age-dependent increase in core amino acid 70 mutants in treatment-naive female patients. Percentage of core wild type (arginine) and mutant amino acid were analyzed as in Figure 5 using only interferon treatment-naive patients. Results for all 561 patients (a), as well as for male (b) and female (c) patients are shown. ND, not determined.

Fig. 2). Wt core 70 was associated with wt core 91, with 40% of patients wt for both core 70 and core 91 and 20% of patients non-wt for both (Fig. 1, $P = 5.4 \times 10^{-9}$). Virus titer did not differ in patients with wt core 70 compared to non-wt when all patients were included (Fig. 3a), but when treatment-naive patients were analyzed separately, virus titer was significantly higher in patients with core 70 wt ($P = 0.02$, Fig. 3c). We found a significant negative linear relationship between virus titer and the number of substitutions in the ISDR ($P < 0.001$, Fig. 3b), regardless of treatment history ($P < 0.001$, Fig. 3d).

Amino acid substitution and age

The proportion of patients with core 70 substitutions increased with age among female patients (Figs 4,5), and the proportion of patients without substitutions in the ISDR tended to increase with age among treatment-naive females ($P = 0.0581$, Fig. 6).

Core 70 a.a. substitution and histological findings

Fibrosis stage and activity were higher in patients with core 70 mutants ($P = 0.028$ and $P = 0.048$, respectively; Fig. 7). There was no apparent correlation between his-

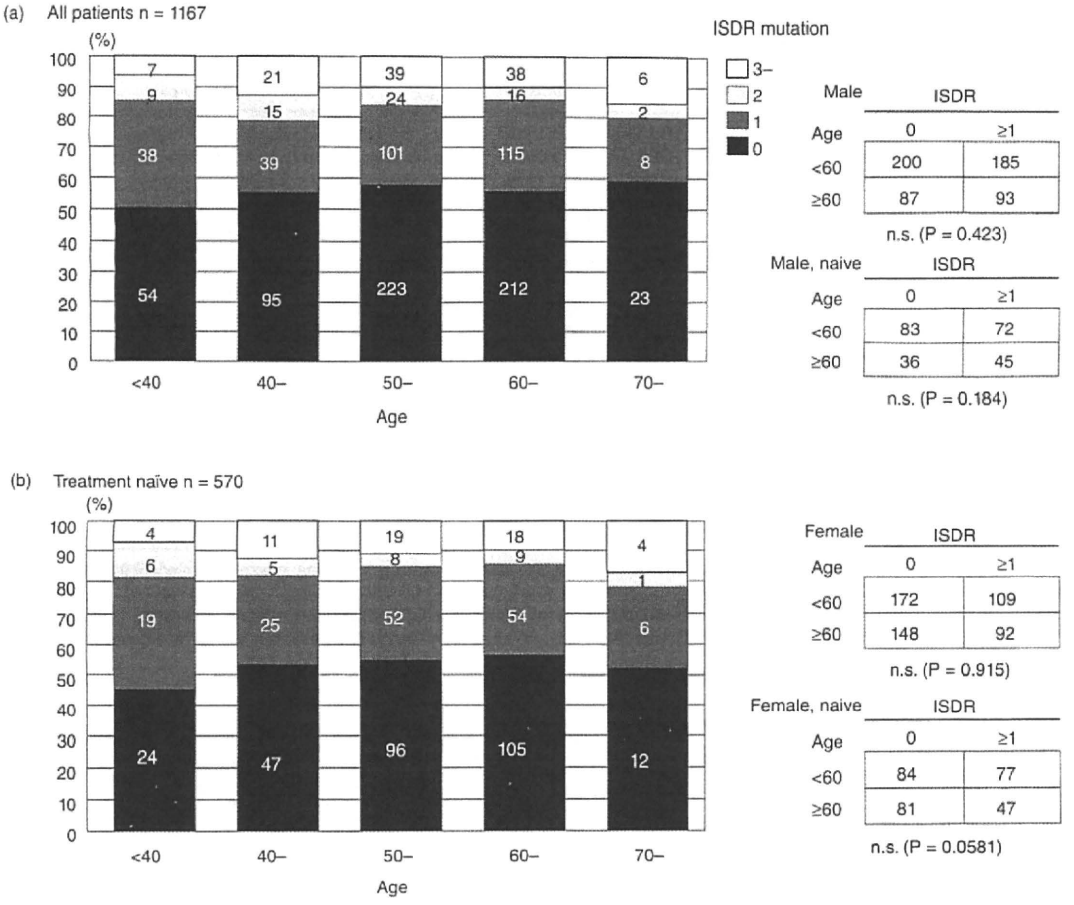


Figure 6 Age-dependent increase in number of amino acid substitutions in the interferon sensitivity determining region (ISDR). The relationship between age and the number of amino acid substitutions in the ISDR was examined. All patients (a) and only naive patients (b) were analyzed. Statistical analysis was performed using the χ^2-test.

tological findings and the number of a.a. substitutions in the ISDR (data not shown).

Correlation between viral a.a. substitutions and clinical conditions

We compared γ-GTP, ALT, LDL cholesterol levels and other clinical conditions between patients with core 70 wild and mutant types (Fig. 8). ALT and γ-GTP levels were significantly higher in patients with core 70 substitutions (Fig. 8a,b). In contrast, LDL cholesterol levels and platelet counts were significantly higher in patients with core 70 wt (Fig. 8c,d). However, only sex, fibrosis, γ-GTP and core 91 substitution were independently associated with core 70 substitution (Table 2). Only viral load and core 70 substitutions are independent predictive factors for the presence of two or more ISDR substitutions (Table 3).

DISCUSSION

WE FOUND THAT factors previously reported to be associated with poor response to IFN-based treatment for chronic hepatitis C tended to be most strongly associated with older female patients. Studies on difficult-to-treat older female patients have so far only been reported in Japan, probably due to the rela-

Table 2 Factors associated with HCV core protein amino acid 70 substitutions

Variable	Simple			Multiple			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age (in 10-year increments)	331	1.1	0.3536				
Sex (male vs female)	365	1.58	0.04178	214	2.09	(1.11–3.95)	0.0234
BMI (kg/m^2)	363	0.763	0.2229				
Diabetes	312	1.77	0.08053				
Fibrosis (F0–1 vs F2–4)	252	2.12	0.007444	214	2.18	(1.15–4.13)	0.017
Activity (A0–1 vs A2–4)	246	1.73	0.04849				
ALT (IU/L)	329	0.866	0.5461				
Platelets (×104/mm^3)	329	0.937	0.7836				
γ-GTP (IU/L)	305	1.69	0.03427	214	1.59	(0.841–3.02)	0.153
Albumin (g/dL)	190	0.765	0.3981				
Fasting blood sugar (mg/dL)	250	0.898	0.6878				
TaqMan PCR (log IU/mL)	327	0.748	0.2232				
HDL cholesterol (mg/dL)	202	1.64	0.1025				
LDL cholesterol (mg/dL)	165	1.25	0.5085				
Total cholesterol (mg/dL)	321	0.907	0.6847				
Core 91 (wild vs others)	365	2.22	0.000393	214	2.68	(1.43–5.02)	0.002
ISDR (0,1 vs >1)	343	1.82	0.03102	214	1.85	(0.853–4)	0.1197

Simple and multiple logistic regression were used to examine the association between substitution at core amino acid 70 and patient and viral factors.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; HDL, high-density lipoprotein; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; ND, not determined; OR, odds ratio.

Table 3 Factors associated with viral ISDR substitutions (0–1 vs >1 mutations)

Variable	Simple			Multiple			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age (in 10-year increments)	311	1	0.9735				
Sex (male vs female)	345	0.644	0.1247				
BMI (kg/m^2)	343	1.14	0.6254				
Diabetes	293	0.818	0.6509				
Fibrosis (F0–1 vs F2–4)	235	1.28	0.4545				
Activity (A0–1 vs A2–4)	229	1.3	0.4281				
ALT (IU/L)	309	1.15	0.646				
Platelets (×104/mm^3)	309	0.668	0.1707				
γ-GTP (IU/L)	287	1.47	0.2115				
Albumin (g/dL)	172	0.979	0.9622				
Fasting blood sugar (mg/dL)	233	1.36	0.3641				
TaqMan PCR (log IU/mL)	307	0.517	0.02527	305	0.529	(0.30–0.95)	0.03223
HDL cholesterol (mg/dL)	189	1.23	0.617				
LDL cholesterol (mg/dL)	152	0.463	0.1199				
Total cholesterol (mg/dL)	303	0.656	0.1537				
Core 70 (wild vs others)	343	1.82	0.03102	305	1.82	(1.01–3.3)	0.04763
Core 91 (wild vs others)	344	0.699	0.2038				

Simple and multiple logistic regression was used to examine the association between the number of substitutions in the ISDR region and patient and viral factors.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; γ-GTP, gamma-glutamyltranspeptidase; HCV, hepatitis C virus; HDL, high-density lipoprotein; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; ND, not determined; OR, odds ratio.

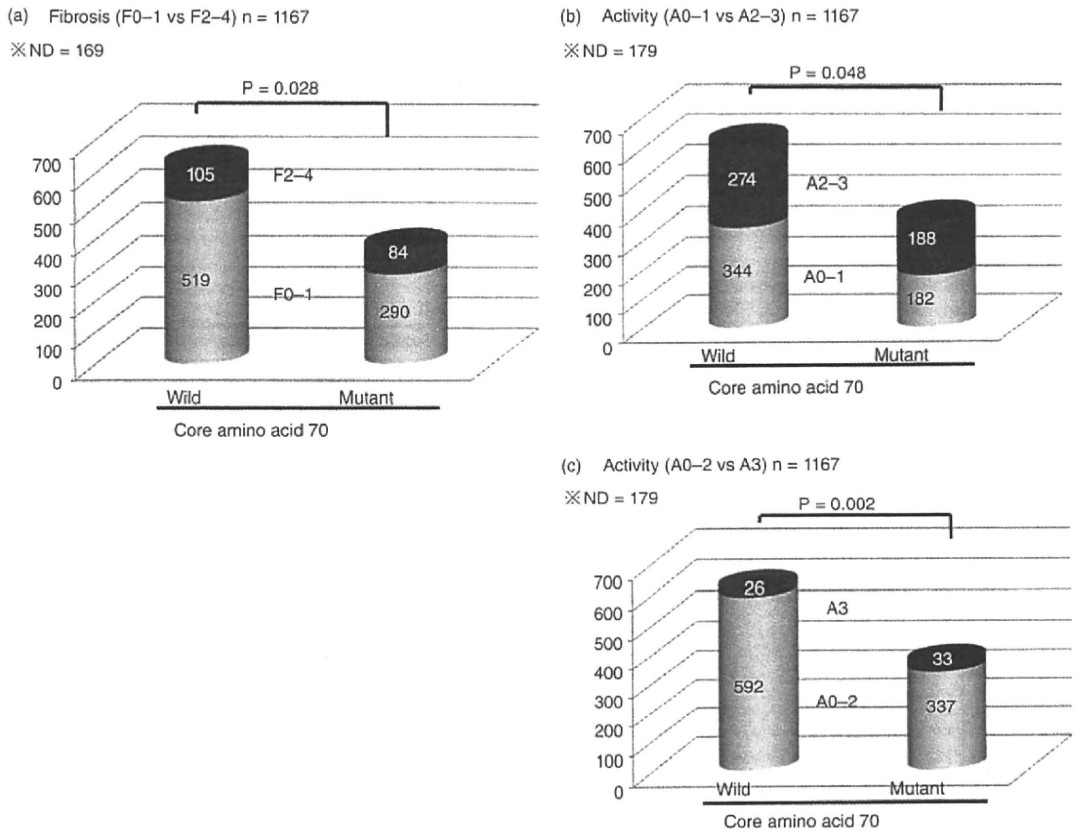


Figure 7 Histological findings and core amino acid 70 substitutions. Relationships between core amino acid 70 (wild type or mutant) and degree of fibrosis (F0–1 and F2–4) (a) and activity (b,c) were examined. Activity was divided into A0–1 and A2–3 (b) or A0–2 and A3 (c) and compared with amino acid 70. ND, not determined.

tively higher age at treatment. The mechanism underlying this association is unknown. Recently, SNP in the IL-28B locus were found to be associated with response to combination therapy as well as to spontaneous eradication of the virus,[42–44] although differences in the eradication rate between men and women have not been reported so far. We have previously reported that incidence of wt core 70 is significantly higher in patients with the IL-28 protective allele.[54] Therefore, it seems reasonable that the wt core 70 confers a selective advantage for the virus in patients with the IL-28 protective allele. During the time when IFN monotherapy was still the standard treatment, female sex, or perhaps the lower iron concentration associated with female sex, had been reported as one of the predictive factors for a favorable response to monotherapy.[55–57] It is possible that spontaneous eradication of the virus occurs during the natural course of chronic hepatitis through IFN produced naturally as a result of liver inflammation in young female patients with wt core 70, resulting in accumulation of core mutant viruses as the patient ages. Further prospective observations are necessary to address this issue.

In this study, we found that each of the previously reported predictive factors that we examined also correlated with HCV a.a. substitutions. Interestingly, a.a. substitutions in the virus are associated with metabolic factors such as LDL and high-density lipoprotein cholesterol and fatty liver-related γ-GTP, and in particular, we found that substitution in the core protein (and possibly ISDR) is correlated with LDL cholesterol. The virus appears to influence expression of genes involved

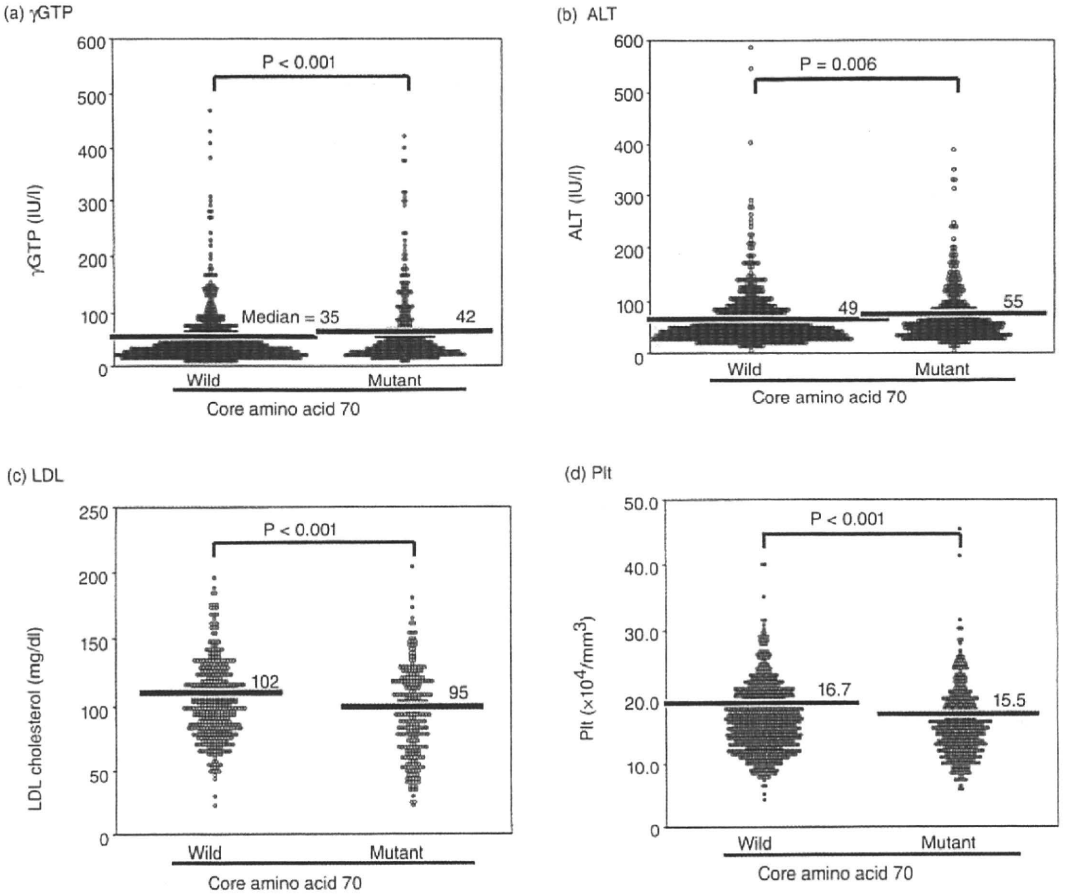


Figure 8 Relationship between blood test findings and core amino acid 70 substitutions. Relationships between core amino acid 70 (wild type or mutant) and gamma-glutamyltranspeptidase (γ-GTP) (a), alanine aminotransferase (ALT) (b), low-density lipoprotein (LDL) cholesterol (c) and platelet count (Plt) (d) were examined. Bars represent the median.

in host cell lipid metabolism to enhance its own replication and secretion.[58] Consequently, metabolic changes induced by infection by different strains of HCV should be investigated further to understand viral mechanisms of IFN resistance and to develop effective personalized therapies.

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