9 Soriano V, Vispo E, Bottecchia M *et al.* Management of hepatitis B virus co-infection on and off antiretroviral therapy. *Curr HIV/AIDS Rep* 2008; **5:** 86–93.
10 Geretti AM, Patel M, Sarfo FS *et al.* Detection of highly prevalent hepatitis B virus coinfection among HIV-seropositive persons in Ghana. *J Clin Microbiol* 2010; **48:** 3223–30.
11 Hadler SC, Judson FN, O'Malley PM *et al.* Outcome of hepatitis B virus infection in homosexual men and its relation to prior human immunodeficiency virus infection. *J Infect Dis* 1991; **163:** 454–59.
12 Thio CL, Seaberg EC, Skolasky R Jr *et al.* HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). *Lancet* 2002; **360:** 1921–26.
13 Spradling PR, Richardson JT, Buchacz K, Moorman AC, Brooks JT. Prevalence of chronic hepatitis B virus infection among patients in the HIV Outpatient Study, 1996–2007. *J Viral Hepat* 2010; **17:** 879–86.
14 Hu KQ. Occult hepatitis B virus infection and its clinical implications. *J Viral Hepat* 2002; **9:** 243–57.
15 Araujo NM, Branco-Vieira M, Silva AC *et al.* Occult hepatitis B virus infection in HIV-infected patients: evaluation of biochemical, virological and molecular parameters. *Hepatol Res* 2008; **38:** 1194–203.
16 Yuan Q, Ou SH, Chen CR *et al.* Molecular characteristics of occult hepatitis B virus from blood donors in southeast China. *J Clin Microbiol* 2010; **48:** 357–62.
17 Pisani E, Garnett GP, Grassly NC *et al.* Back to basics in HIV prevention: focus on exposure. *BMJ* 2003; **326:** 1384–87.
18 Prabawanti C, Bollen L, Palupy R *et al.* HIV, sexually transmitted infections, and sexual risk behavior among transgenders in Indonesia. *AIDS Behav* 2011; **15:** 663–73.
19 Nelwan EJ, Van Crevel R, Alisjahbana B *et al.* Human immunodeficiency virus, hepatitis B and hepatitis C in an Indonesian prison: prevalence, risk factors and implications of HIV screening. *Trop Med Int Health* 2010; **15:** 1491–98.
20 Sugauchi F, Mizokami M, Orito E *et al.* A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* 2001; **82:** 883–92.
21 Iacomi F, Vincenti D, Vairo F *et al.* Effect of HIV co-infection on mutation patterns of HBV in patients with lamivudine-resistant chronic hepatitis B. *J Med Virol* 2009; **81:** 1151–56.
22 Abe A, Inoue K, Tanaka T *et al.* Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol* 1999; **37:** 2899–903.
23 Jilg W, Sieger E, Zachoval R, Schätzl H. Individuals with antibodies against hepatitis B core antigen as the only serological marker for hepatitis B infection: high percentage of carriers of hepatitis B and C virus. *J Hepatol* 1995; **23:** 14–20.
24 Thio CL, Netski DM, Myung J, Seaberg EC, Thomas DL. Changes in hepatitis B virus DNA levels with acute HIV infection. *Clin Infect Dis* 2004; **38:** 1024–29.
25 Shire NJ, Rouster SD, Stanford SD *et al.* The prevalence and significance of occult hepatitis B virus in a prospective cohort of HIV-infected patients. *J Acquir Immune Defic Syndr* 2007; **44:** 309–14.
26 Bagaglio S, Bianchi G, Danise A *et al.* Longitudinal evaluation of occult hepatitis B infection in HIV-1 infected individuals during highly active antiretroviral treatment interruption and after HAART resumption. *Infection* 2011; **39:** 121–26.
27 Cao GW. Clinical relevance and public health significance of hepatitis B virus genomic variations. *World J Gastroenterol* 2009; **15:** 5761–69.
28 Idoko J, Meloni S, Muazu M *et al.* Impact of hepatitis B virus infection on human immunodeficiency virus response to antiretroviral therapy in Nigeria. *Clin Infect Dis* 2009; **49:** 1268–73.
29 Thedja MD, Roni M, Harahap AR, Siregar NC, Ie SI, Muljono DH. Occult hepatitis B in blood donors in Indonesia: altered antigenicity of the hepatitis B virus surface protein. *Hepatol Int* 2010; **4:** 608–14.
30 Attia KA, Eholié S, Messou E *et al.* Prevalence and virological profiles of hepatitis B infection in human immunodeficiency virus patients. *World J Hepatol* 2012; **4:** 218–23.
31 Pourkarim MR, Lemey P, Amini-Bavil-Olyaee S *et al.* Molecular characterization of hepatitis B virus strains circulating in Belgian patients co-infected with HIV and HBV: overt and occult infection. *J Med Virol* 2011; **83:** 1876–84.
32 UNAIDS. Report on the Global AIDS epidemic 2008. Available at: http://data.unaids.org/pub/GlobalReport/2008/jc1511_gr08_executivesummary_en.pdf#search='ReportontheGlobalAIDSEpidemic2008'. Accessed June 6, 2011.
33 Wisaksana R, Indrati AK, Fibriani A *et al.* Response to first-line antiretroviral treatment among human immunodeficiency virus-infected patients with and without a history of injecting drug use in Indonesia. *Addiction* 2010; **105:** 1055–61.
34 Sidiq H, Ankoma-Sey V. HIV-related liver disease: infections versus drugs. *Gastroenterol Clin North Am* 2006; **35:** 487–505.
35 Gaglio PJ, Sterling R, Daniels E, Tedaldi E. Hepatitis B virus and HIV coinfection: results of a survey on treatment practices and recommendations for therapy. *Clin Infect Dis* 2007; **45:** 618–23.

SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article:

Table S1 HBV viral load, AST, and ALT in HBsAg-positive patients according to CD4 cell counts.

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＜原　著＞

C型慢性肝炎時から肝癌発生まで経過を追えたC型肝炎ウイルスジェノタイプ1bのコア蛋白アミノ酸多様性と肝癌発生との関連性

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要旨：C型肝炎ウイルスによるC型慢性肝炎から肝癌発生にはウイルス側と宿主側の因子が関与していると考えられる．ウイルス側の因子としてはコア蛋白70番目および91番目のアミノ酸変異が報告されている．今回我々は，C型慢性肝炎時より肝癌発生まで平均7年以上経過を追えた49例の症例と，同時代に経過観察が可能でかつ肝癌を発生しなかった症例50例を対象にこれらコア蛋白のアミノ酸多様性の違いを明らかにすると同時に，肝癌発生前後での多様性の変化につき検討した．その結果，コア蛋白70番目のアミノ酸変異が肝癌発生に関与しているが，91番目のアミノ酸は関与していないことが示唆された．また，肝癌発生群にて肝癌発生前後におけるコア蛋白アミノ酸の多様性の変化は少なく，この領域は極めて保存性が高いと考えられた．多変量解析ではコア蛋白70番目のアミノ酸Glnとαフェトプロテイン（AFP）値が有意に肝癌と関連していた．

索引用語：　C型肝炎ウイルス　慢性肝炎　肝癌
コア蛋白70番目および91番目のアミノ酸変異　累積肝癌発生率

緒　言

C型肝炎ウイルス（HCV）はC型慢性肝炎，肝硬変，肝癌の原因となることはよく知られている[1)~3)]．インターフェロン（IFN）を基本とした治療はウイルスを排除し，著効例では劇的に生化学的，組織学的に改善し肝癌のリスクも低下する[4)]．しかし，治療に反応しなかった例では炎症が持続し肝硬変，肝癌へ進行する．

IFNに対する反応性には，宿主側の因子では男性，高齢，肝線維化の進展，IL28Bの遺伝子変異などが関与し[5)~8)]，ウイルス側の因子としてコア蛋白70番目と91番目のアミノ酸変異，及びNS5AのIRRDR（interferon/ribavirin resistance determining region）やISDR（interferon sensitivity determining region）の変異数などが関与することが報告されている[9)~12)]．

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肝癌発生には，ウイルス側と宿主側の因子が複雑に絡み合っていると考えられる．IFNに対する反応性も肝癌発生に寄与することが報告されており，上記のIFN反応性に関する因子は肝癌発生にも関与する．少なくとも10種類のHCV蛋白の中でコア蛋白，NS3，NS5Aが肝癌発生に関与することが培養細胞系やトランスジェニックマウス等を用いた動物実験系で報告されている[13)]．臨床的にはコア蛋白70番目，91番目のアミノ酸変異が肝癌発生に関与しているという報告がなされている[14)~16)]．

今回我々は，C型慢性肝炎時から肝癌発生にいたるまで長期間にわたり経過観察可能であり，かつC型慢性肝炎時と肝癌発生時点でのペア血清がある症例49例を対象に，C型肝炎ウイルスコア蛋白のアミノ酸がどのように変化するのか，またどのように肝癌発生に関与するのかを，同時代に10年以上経過観察可能でかつ肝癌を発生しなかった症例50例をコントロールとして比較検討した．

対象および方法

患者

対象は1988年から2003年の間に明石市立市民病院

Table 1　Clinical characteristics studied.

	HCC group	Control	P value
No.	49	50	
Age (y/o)	57±7.0*	56±8.7	0.2465
Sex (M：F)	31：18	35：14	
ALT (IU/l)	160±80	132±51	0.009
AST (IU/l)	113±62	89±44	0.02
PLT count (×104/ul)	16±2.8	16±2.2	0.7766
AFP (ug/L)	29±33	18±4.6	0.002
Grading score	8.7±0.9	8.3±1.3	0.0454
Staging score	2.4±0.5	2.2±0.5	0.0305
Level of viremia (KIU/ml)	593±112	605±94	0.0914

*Mean±S.D.

肝臓内科を訪れ，初診時C型慢性肝炎と組織学的に診断され，その後肝癌発生するまで平均6.5±2.9年（3-15年）経過を追うことができた肝癌症例（HCC群）49例である．いずれも初診時と肝癌発生時の血清（ペア血清）が保存されており，下記の解析に用いた．初診時，C型慢性肝炎と診断されIFN治療を受ける前の血清サンプルをpre HCC sampleとし，肝癌診断時における血清サンプルをpost HCC sampleとした．HCVのジェノタイプは全例1b型であった．コントロール群（non-HCC群）として，1988年から2003年の間に当科を訪れ，初診時組織学的にC型慢性肝炎と診断され10年間以上（10-15年）経過を追え，かつHCC群49例と平均年齢を合わせた50例を用いた．いずれも初診時にIFN治療を受ける前に血清サンプル（non-HCC serum sample）があり，かつ全例HCVジェノタイプ1b型の症例を選んだ．全ての患者はHCV抗体，HCV RNAいずれも陽性であり，B型慢性肝炎，自己免疫性肝炎は除外した．全例，初診時から数カ月以内にIFN単独療法（Sumiferon（大日本住友製薬，東京，日本），Intron A（MSD，東京，日本）を600-1000万単位/日，週3回投与，6カ月間）を1回のみ受けているが，いずれもウイルス駆除に至った症例はなかった．IFN治療後は強力ミノファーゲンやウルソ，グリチロンの内服を受けていた．患者は経過観察中2カ月に1回診察に訪れており，血液検査を受け，肝癌の有無を調べるため6カ月から1年ごとに腹部エコー，CTスキャンを受けていた．

全ての患者から肝生検，血清保存，治療に対する同意書が得られており，明石市立市民病院及び神戸大学大学院医学研究科の倫理委員会にて承認を得た．

HCVコア蛋白70番目及び91番目のアミノ酸残基の解析

HCV RNAは140μlの血清から市販のキット（QIAmp viral RNA kit，QIAGEN，Tokyo，Japan）を用いて抽出し，コア遺伝子領域をRT-PCR法を用いて増幅し，増幅されたcDNAの塩基配列をdirect sequence法にて決定した[11]．アミノ酸配列はGENETYX Win software version 7.0（GENETYX Corp.，Tokyo，Japan）を用いて決定した．HCVコア蛋白のアミノ酸残基の番号はジェノタイプ1bのプロトタイプであるHCV-Jに従った．

統計学的解析

統計学的解析はStudent t test，Fisher's exact probability testあるいはChi square testを用いて行った．単変量解析で<0.1の因子を選び多変量解析を行った．得られたデータはLog-rank testにて評価した．全ての解析はSPSS version 16 software（SPSS Inc.，Chicago，IL）を用いて行った．P<0.05を有意とした．

結　果

1）HCC群と対照（non-HCC）群の臨床的特徴

初診時のIFN治療前のHCC群及びnon-HCC群の臨床的特徴を比較した（Table 1）．HCC群の方が有意にALT値，AST値，組織学的grading，staging値，AFP値が高値であった（各々p=0.0411，0.042，0.0455，0.0305と0.002）．

2）HCC群と対照（non-HCC）群におけるHCVコア蛋白70番目と91番目アミノ酸の比較検討

HCC群とnon-HCC群におけるHCVコア蛋白70番目と91番目のアミノ酸を比較検討した（Table 2）．

Table 2 Correlation between core polymorphism and HCC

Factor	Total	HCC	Non-HCC	*P* value
Mutant core (Gln^{70}/Met^{91}, Gln^{70}/Leu^{91} and Arg^{70}/Met^{91})	49	27 (55%)	22 (45%)	
Wild core (Arg^{70}/Leu^{91})	48	22 (46%)	26 (54%)	0.4
Gln^{70}	28	21 (75%)	7 (25%)	
Non-Gln^{70}	70	28 (40%)	42 (60%)	0.003
Non-Leu^{91}	28	12 (43%)	16 (57%)	
Leu^{91}	70	37 (53%)	33 (47%)	0.5

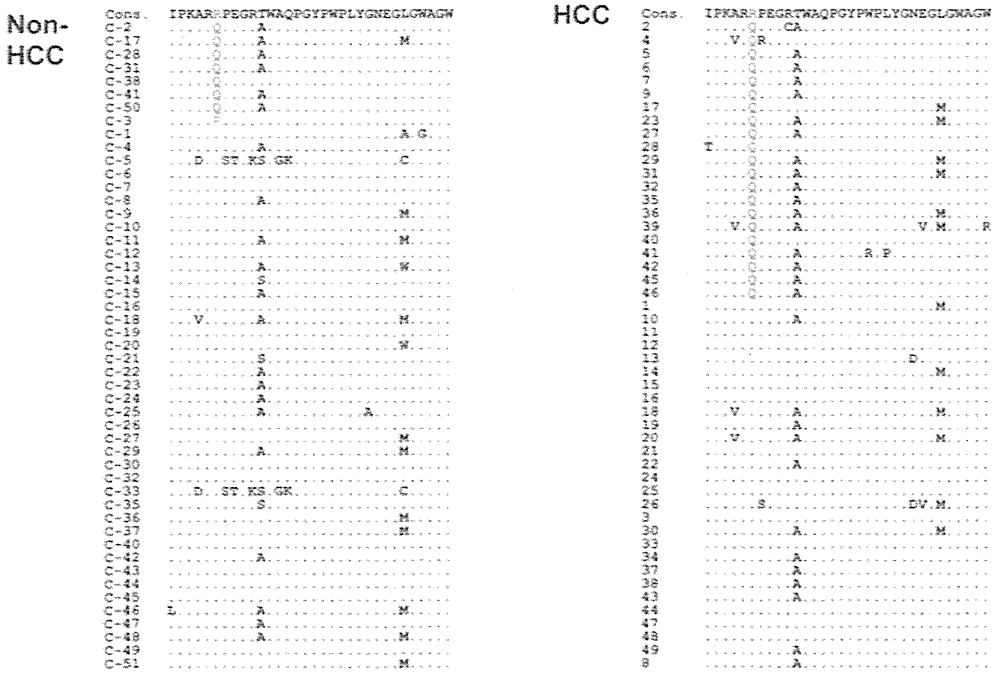

Fig. 1 Comparison in the polymorphism of the core protein of HCV genotype 1b between HCC and the control (non-HCC) groups. Serum samples examined were obtained before IFN treatment in all patients of both groups.

解析した血清はHCC群，non-HCC群いずれも，全て初診時，IFN治療前のものである．コア蛋白70番目のアミノ酸残基はGlnが，また，91番目はMetが変異型である．

70番目と91番目の両方あるいはいずれか一方に変異を有するコア蛋白は49例に見られたが，そのうちの27例（55%）はHCC群　に，22例（45%）はnon-HCC群に見られた．

一方，70番目のアミノ酸の変異のみに着目すると，Gln^{70}は28例に見られ，そのうちの21例(75%)がHCC群で見られた．non-HCC群ではGln^{70}は7例（25%）のみであり，両群間の違いは有意であった（$p=0.003$）．

91番目のアミノ酸の変異については，Met^{91}は28例に見られ，そのうちの12例（43%）がHCC群，16例（57%）がnon-HCC群であり，両群間に有意差は認められなかった．

個々の症例のコア蛋白の当該領域のアミノ酸配列をFig. 1に示す．

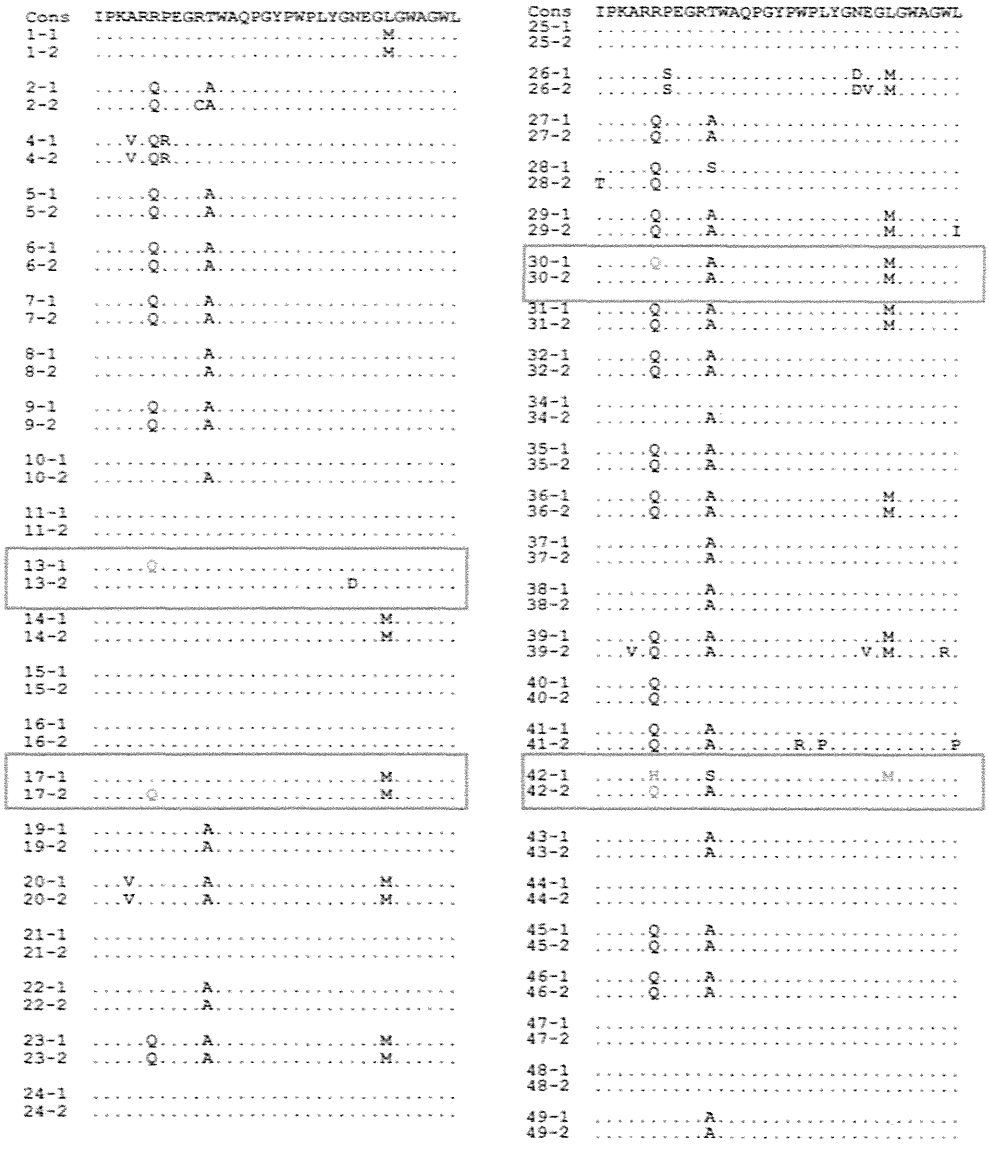

Fig. 2 Sequence patterns of the HCV core protein at positions 70 and 91 before and after the occurrence of HCC. The number 1 followed by patient number indicates the sample before the occurrence of HCC and the number 2 after the occurrence of HCC. Serum samples before HCC were obtained before the IFN treatment.

上記のように，HCC群とnon-HCC群で唯一有意差を認めたのは，HCC群でコア蛋白のアミノ酸残基70番目のGln変異の頻度が高いということだけであった．そこで，HCC群のなかで，70番目のアミノ酸残基がGlnである症例とArgである症例の臨床的特徴を比較したが，両群間に有意差を認めなかった（data not shown）．

3）HCC発生前後でのHCVコア蛋白70，91番目のアミノ酸残基の変化

HCC群で，肝癌発生前後のコアアミノ酸残基を比較した（Fig. 2）．患者番号の後の記載されている数字1はHCC発生前の初診時，IFN治療前のC型慢性肝炎時点での血清であり，数字2はHCC診断時の血清である．

Table 3 Univariate and multivariate regression analyses to indentify factors associated with HCC

Variable	Univariate		Multivariate	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Core-Gln^{70}	0.25 (0.11-0.55)	0.003	6.8 (2.1-23.0)	0.003
AFP (>20 ug/L)	13 (5.2-30.3)	0.002	19.7 (4.9-80.2)	0.001
ALT (>165 IU/L)	4.1 (1.8-8.7)	0.006		
AST (>65 IU/L)	3.9 (1.5-10.0)	0.003		
Fibrosis staging score (≥3)	2.4 (1.1-4.9)	0.02		

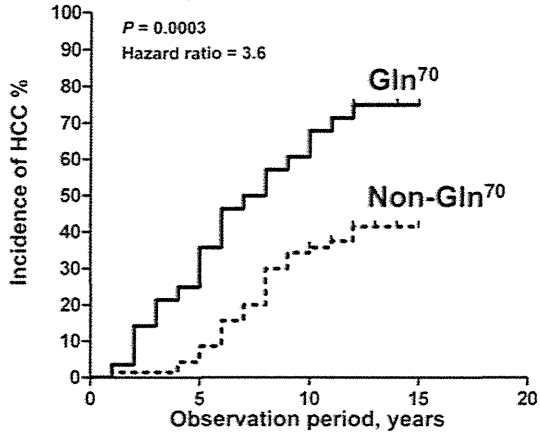

Fig. 3 Cumulative HCC occurrence rates based on the sequence pattern of the HCV core protein at position 70. In follow up study, the cumulative HCC rates of patients infected with HCV isolates with core protein of *Gln*^{70} and *non-Gln*^{70} were compared using the pre HCC samples in the HCC and the control groups at the first visit. All samples were obtained before IFN treatment. The rates were 36% vs 9% at the end of 5 years; 68% vs 36% at the end of 10 years; and 75% vs 41% at the end of 15 years. They were significantly different between the two groups ($P = 0.0001$; Log-rank test).

45例のHCC症例で4症例（0.9%）のみに変化が見つかった．患者番号13には2カ所，患者番号17には1カ所，患者番号30には1カ所，患者番号42には3カ所変化が認められた．これらの変化には共通なものや有意なものは認められなかった．

4）単変量および多変量解析

単変量解析により，HCCと有意の相関を示す因子としてHCVコア蛋白のGln^{70}変異，AFP高値，ALT高値，AST高値及び肝の線維化スコアが抽出された（Table 3）．これらについて多変量解析を行ったところ，コア蛋白のGln^{70}変異（p＝0.003）とAFP高値（p＝0.001）が独立してHCCと相関することがわかった．

5）HCVコア蛋白アミノ酸70番目のGln変異に基づく累積肝癌発生率

HCC群において，初診時，IFN治療前の血清サンプルで，コア蛋白アミノ酸70番目がGlnに変異している患者（Gln^{70}群）と変異していない患者（non-Gln^{70}群）における15年間での累積肝癌発生率を比較検討した(Fig. 3)．5年間の累積肝癌発生頻度はGln^{70}群で36%，non-Gln^{70}群で9%であり，また10年間の累積肝癌発生頻度はGln^{70}群で68%，non-Gln^{70}群で36%，15年間では75% vs 41%であった．Glnに変異している患者群で有意に肝癌を発生する頻度が高かった（p＝0.0001；Lon-rank test)．

考　察

肝癌発生には宿主側とウイルス側の因子が複雑に絡み合っていると考えられている．HCVジェノタイプ1b，C型慢性肝癌において，コア蛋白が肝癌発生に関与しているとの報告がなされている[13)~16)18)~20)]．とりわけコア蛋白の70番目と91番目のアミノ酸変異が肝癌発生に寄与しているといわれている[13)~16)]．今回の我々の研究からもコア蛋白70番目のアミノ酸がGlnに変異していること（Gln^{70}）が肝癌発生に有意に関与しているという結果が得られた．しかし，91番目のアミノ酸変異との有意な関連は認められなかった．

Kobayashiら[15)]は，70番目のアミノ酸が年月を経ると同時にArgからGlnに変化し病変の重篤度を変えるか，あるいは元々これらGlnに変異しているウイルス株が重篤な肝疾患を引き起こすのかは，一時期のみの血清からの測定検査からは結論付けることは難しいと述べている．Nakamotoら[16)]もまたnon-double-wild typeが肝疾患の重篤度を増幅させ肝癌発生に関与している

ことを示唆しているが，いずれの論文も一時期のみの血清を測定した結果からの結論であり，経過観察の血清検査を必要としている．今回の我々の論文の興味深い点は，まさにこの時間の経過によりアミノ酸変異がおこり肝癌へ移行するのか，元々から重篤度の高い肝疾患を引き起こし肝癌発生に寄与するウイルス株に感染しているのかという問いに対する答えが肝癌発生前後のペア血清の解析によって明らかにされたところにある．即ち，HCVコア蛋白のアミノ酸残基は調べ得たHCC群では極めて保存性が高く，時間の経過とともに変わりうるものではないことから，むしろ重篤度の高い肝疾患を引き起こし肝癌発生に深く関与するウイルス株に元々感染している可能性が高いことが示唆された．変異を起こした4例ではIFN治療によりもたらされた可能性も否定できないが，加藤らによりPegIFN/RBV治療により，コア蛋白70位の変異はおこらないことを示唆することが発表されており，その可能性は極めて低いと考えられる[17]．コア蛋白アミノ酸配列の経時的保存性に関しては，今回はHCC群のみにしかペア血清がないことからコントロール群では調べられていないため，今後の検討が必要であろう．

なぜHCVコア蛋白の70番目のアミノ酸が変化することが肝癌発生に関与するのか，すなわち上記のように単に重篤度の高い肝疾患を引き起こすためだけなのか，あるいは他にも理由があるのかは完全に解明されていない．コア蛋白はRas癌遺伝子とともに働きラット胎児を悪性形質転換させると報告されている[18]．また，Pavioら[19]はコア蛋白がSmad3とともに働き，TGF-β経路を阻害するとも報告している．Delihemら[20]は，HCC患者から得られたコア蛋白の70番目と91番目がnon-wild typeである場合は肝癌発生に関与するPKRを活性化すると報告している．未だ議論のあるところであり，今後の解明が待たれる．

多変量解析では，調べたウイルス側因子ではコア蛋白70番目のGlnのみが有意にHCC発生に相関していた．また，臨床的にはAFP値が有意差に相関していた．

以上結語として，本研究より，1）HCVコア蛋白のアミノ酸70番目のGlnが有意に肝癌発生に相関していたが，91番目の変異は相関していなかった2）HCC群において肝癌発生前後におけるコアアミノ酸残基の変化を調べた結果，極めて保存性が高かった．3）AFP値は有意に肝癌発生に相関していることが示唆された．

今後，HCC発生に関与するウイルス因子として，コア蛋白のみならず，その他の領域に関しても同様の解析が必要と考えられた．

文　献

1) Alberti A, Chemello L, Benvegnu L. Natural history of hepatitis C. J Hepatol 1999; 31: S17—24
2) Seef LB. Natural history of chronic hepatisis C. Hepatology 2002; 36: S35—S46
3) Simmonds P. Variability of hepatitis C virus. Hepatology 1995; 21: 570—583
4) Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma : national surveillance program of chronic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study group. Inhibition of hepatocarcinogenesis by interferon therapy. Ann Intern Med 1999; 131: 174—181
5) Lok AS, Seelf LB, Morgan TR, et al. Incidence of hepatocellular carcinoma and associated risk factors in hepatitis Crelated advanced liver disease. Gastroenterology 2009; 136: 138—148
6) Ge D, Fellary J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009; 461: 399—401
7) Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009; 41: 1105—1109
8) Supplah V, Moldovan N, Athlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon alpha and ribavirin therapy. Nat Genet 2009; 41: 1100—1104
9) Akuta N, Suzuki F, Kawamura Y, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005; 48: 372—380
10) Akuta N, Suzuki F, Kawamura Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b:Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007; 46: 403—410
11) El-Shamy A, Nagano-Fujii M, Sasase N, et al. Sequence variation in hepatitis C virus nonstructural

protein 5A clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 2008; 48: 38—47

12) Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996; 334: 77—81
13) Banerjee A, Ray RB, Ray R. Oncogenic potential of hepatitis C virus proteins. Viruses 2010; 2: 2108—2133
14) Akuta N, Suzuki F, Kawamura Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology 2007; 46: 1357—1364
15) Kobayashi M, Akuta N, Suzuki F, et al. Influence of amino-acid polymorphism in the core protein on progression of liver disease in patients infected with hepatitis C virus genotype 1b. J Med Virol 2010; 82: 41—48
16) Nakamoto S, Imazeki F, Fukai K, et al. Association between mutations in the core region of hepatitis C virus genotype 1 and hepatocellular carcinoma development. J Hepatol 2010; 52: 72—78
17) 加藤直也, 李ウェンウェン, 小池和彦. ペグインターフェロン・リバビリン併用療法におけるC型肝炎ウイルスコア蛋白第70番目アミノ酸野生型と変異型のダイナミクスからわかったこと. 肝臓　2011；52（Suppl. 1）：A145
18) Ray RB, Lagging LM, Meyer K, et al. Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorgenic phenotype. J Virol 1996; 70: 4438—4443
19) Pavio N, Battaglia S, Boucreux D, et al. Hepatitis C virus core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with 5mad3 and inhibit the TGF-beta pathway. Oncogene 2005; 24: 6119—6132
20) Delhem N, Sabile A, Gajardo R, et al. Activation of the interferon-inducible protein kinase PKR by hepatocellular carcinoma derived-hepatitis C virus core protein. Oncogene 2001; 20: 5836—5845

The relationship between the core protein polymorphism and the development of hepatocellular carcinoma in patients chronically infected with hepatitis C virus genotype 1b

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The aim of this study was to determine whether amino acid residues 70 and 91 of the core protein of hepatitis C virus (HCV) genotype 1b are associated with the development of hepatocellular carcinoma (HCC), and whether this region could change over time with the development of HCC. A total of 49 paired serum samples from patients who had been followed up for more than 7 years from the time of chronic hepatitis C (pre HCC sample) before IFN treatment until the time of the development of HCC (post HCC samples) were examined for mutations in the core protein of HCV. A total of 50 sera from 50 pre-IFN treatment chronic hepatitis C patients who also had been followed up for more than 10 years and did not develop HCC were used as a control.

The incidence of the HCV core protein mutation at position 70 (Gln^{70}) was significantly higher in the HCC group than in the control ($p = 0.003$), while the residue at 91 (non-Leu^{91}) did not significantly differ between the two groups. Sequence patterns of the core protein at positions 70 and 91 did not significantly change over time before and after the occurrence of HCC in the HCC group. The present study suggests that the residue of the HCV core protein at position 70, but not 91, was significantly associated with the development of HCC, and that the residue did not change over time with the development of HCC.

Key words: hepatitis C virus　chronic hepatitis C　hepatocellular carcinoma
mutation of the HCV core protein at positions 70 and 91　cumulative HCC rates

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Isolation of Human Monoclonal Antibodies to the Envelope E2 Protein of Hepatitis C Virus and Their Characterization

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Abstract

We isolated and characterized two human monoclonal antibodies to the envelope E2 protein of hepatitis C virus (HCV). Lymphoblastoid cell lines stably producing antibodies were obtained by immortalizing peripheral blood mononuclear cells of a patient with chronic hepatitis C using Epstein-Barr virus. Screening for antibody-positive clones was carried out by immunofluorescence with Huh7 cells expressing the E2 protein of HCV strain H (genotype 1a) isolated from the same patient. Isotype of resulting antibodies, #37 and #55, was IgG1/kappa and IgG1/lambda, respectively. Epitope mapping revealed that #37 and #55 recognize conformational epitopes spanning amino acids 429 to 652 and 508 to 607, respectively. By immunofluorescence using virus-infected Huh7.5 cells as targets both antibodies were reactive with all of the nine different HCV genotypes/subtypes tested. The antibodies showed a different pattern of immuno-staining; while #37 gave granular reactions mostly located in the periphery of the nucleus, #55 gave diffuse staining throughout the cytoplasm. Both antibodies were shown by immuno-gold electron microscopy to bind to intact viral particles. In a neutralization assay (focus-forming unit reduction using chimeric infectious HCV containing structural proteins derived from genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a, and 7a), #55 inhibited the infection of all HCV genotypes tested but genotype 7a to a lesser extent. #37 did not neutralize any of these viruses. As a broadly cross-neutralizing human antibody, #55 may be useful for passive immunotherapy of HCV infection.

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Introduction

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family and contains a 9.6 kb positive-strand RNA genome. The virus has been classified into seven major genotypes. The envelope glycoproteins, E1 and E2, mediate viral entry via cellular co-receptors, including CD81, claudin-1, occludin, and SBR1. The E1 and E2 proteins, located on the surface of viral particles, are the potential targets of neutralizing antibodies. At present, however, neither antibody-based prophylaxis nor an effective vaccine is available.

HCV persists in the presence of circulating antibodies. It has been speculated that this relates to the highly mutable, quasispecies nature of this RNA virus and the continual emergence of neutralization-resistant strains. However, the persistence of HCV in the presence of anti-HCV antibodies can not be fully explained by high variability alone. It has been found that neutralizing activity is detectable in sera from infected patients during both acute and persistent HCV infection [1,2], and that high titers of neutralizing antibodies correlate with natural resolution of chronic hepatitis C [3]. Further, polyclonal hyper-immune antibodies to the E2 protein have been shown to prevent or delay the onset of HCV infection in chimpanzees when administrated before exposure to the virus [4]. The ability of HCV to persist in its host despite the presence of neutralizing antibodies remains unexplained.

With the advent of recently developed systems to study the full cycle of HCV infection [5], various human monoclonal antibodies to the E1 and E2 proteins have been evaluated for their neutralizing activity and some of them were found to contain broadly cross-neutralizing antibodies [6–11]. Passive immunotherapy with such antibodies has preventive and therapeutic potential particularly for preventing HCV re-infection in liver transplant recipients.

During the course of our studies on lymphoblastoid cell lines producing antibodies against HCV, we were able to isolate one clone producing broadly cross-neutralizing antibodies and one

clone producing non-neutralizing antibodies from a well-characterized HCV-carrier (patient H). Isolation and characterization of these human monoclonal antibodies are detailed in this report.

Materials and Methods

Peripheral Blood Mononuclear Cells (PBMC) and Cell Lines

Following written informed consent, the blood sample was obtained in 2000 from patient H who developed chronic HCV infection after transfusion in 1977 [12]. The work was conducted with approval from the Institutional Review Board of the Clinical Center, National Institutes of Health, Bethesda, USA. (IRB # 91-CC-0117). PBMC were isolated by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden), washed three times in phosphate-buffered saline (PBS), re-suspended in Cell Culture Freezing Medium (Life Technologies Japan, Tokyo, Japan), and stored at −80°C until use. Huh 7 cells, a cell line derived from a hepatocellular carcinoma, and highly permissive Huh7.5 cells [13] (provided by C. Rice, Rockefeller University, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Nichirei, Tokyo, Japan). Cells were grown at 37°C in a CO_2 incubator.

Immunofluorescence (IF)

After fixation in ice-cold 100% acetone for 5 min, cells were incubated with primary antibody for 30 min at room temperature, washed 3 times in PBS, and incubated with a 1:200 dilution of the AlexaFluor 488 (Invitrogen, Carlsbad, CA, USA) secondary antibody for 30 min at room temperature. The samples were examined under a TE200 fluorescence microscope (Nikon, Tokyo, Japan).

Equilibrium Centrifugation in Sucrose Density Gradient (SDG)

A crude supernatant containing HCV was centrifuged at 2,380×g for 15 min at 4°C, filtered through the 0.45 μm membrane, concentrated approximately 100-fold using Amicon Ultra-15 centrifugal filter unit with Ultracel-100 (100 kD cut-off) membrane (Millipore, Billerica, MA, USA). The concentrated sample (1.5 ml) was overlaid on 6 ml of a discontinuous gradient with 10, 20, 30, 40, 50, and 60% (w/v) sucrose steps and centrifuged at 289,000×g for 20 h at 4°C in a CS 100GXL centrifuge (Hitachi, Tokyo, Japan). Buoyant density of fractions was determined by refractometry and expressed in g/ml.

Immuno-gold Electron Microscopy (EM)

For preparing a concentrated virus sample, fraction 3 obtained from the SDG centrifugation described above was diluted in 6.5 ml PBS and spun down at 215,000×g for 4 h at 4°C in a S58A-0015 rotor (Hitachi, Tokyo, Japan). The resulting pellet was suspended in 50 μl of PBS, mixed with an equal volume of antibody #55, #37, or a control antibody (500 μg/ml), and incubated overnight at 4°C. The mixture was then treated with 10 μl of goat anti-human IgG conjugated with colloidal gold-particles (Jackson Labs, Grove, PA, USA) overnight at 4°C. The sample was placed on a high resolution carbon grid, STEM100Cu (Oken, Tokyo, Japan), negatively stained with 2% uranyl acetate solution, and examined under a JEM-100C transmission electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Reverse-transcription (RT), PCR, and Quantitative PCR (qPCR)

Extraction of RNA, RT, and PCR were carried out as described previously [14]. The amount of HCV cDNA was measured by qPCR using SYBR Premix Ex Taq (Takara, Tokyo, Japan) with an ABI Prism model Fast 7700 instrument (Applied Biosystems, Tokyo, Japan). To determine copy numbers, standard curves were prepared with serial 10-fold dilutions of a known amount of a plasmid bearing the amplified HCV sequence. We used primers that amplified the 5′ non-coding region of the viral genome. The sequences of the primers used were 5′-TTC ACG CAG AAA GCG TCT AG-3′ as a sense primer and 5′-CCC TAT CAG GCA GTA CCA CA-3′ as an anti-sense primer [15]. For detection of RNA encoding the V_H regions of antibodies, we used primer pair CG1z (5′-GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG-3′) and VH6a (5′-CAG GTA CAG CTC GAG CAG TCA GG-3′) for #37, and primer pair CG1z and VH3a (5′-GAG GTG CAG CTC GAG GAG TCT GGG-3′) for #55. The RT-PCR products were cloned into pCR4TOPO (Invitrogen, Carlsbad, CA, USA) and the molecular clones were sequenced with an ABI PRISM^{TM}310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Expression of HCV E2 Proteins

Forns et al., [16] reported that the HCV E2 protein, when expressed on the cell surface, acquired its native conformation more efficiently when truncated at amino acid (aa) 661 of the viral genome. Therefore, we prepared expression vectors encoding truncated forms of E2 (aa 384 to 661) derived from HCV isolates of patient H, obtained in 1977 (strain H77, AF011751) and in 2000 (strain H00) for the screening of antibody-positive clones. For epitope mapping, we prepared vectors encoding various sizes of E2 proteins derived from strain H77. The inserts were amplified by RT-PCR, cloned into pDisplay (Invitrogen, Carlsbad, CA, USA) in frame between a signal sequence and a trans-membrane domain. All clones were sequenced to ensure that the DNA encoded the authentic HCV sequence. Huh 7 cells were grown in Lab-Tek 8-chamber slides (Nagle Nunc, Naperville, IL, USA) until 80% confluent and transfected with the constructs using SuperFect (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After 48 h, cells were washed, fixed with cold acetone for 5 min, and stored at −80°C until use. Expression of the E2 proteins was verified by IF with rabbit hyperimmune sera raised against various domains of HCV E2.

HCV Plasmids and Generation of Infectious HCV

Plasmid pJFH1 that contains full-length cDNA of HCV strain JFH1 was provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) [5]. Plasmids pFK-JFH/Con1/C-842-dg, pFK-JFH/J6/C846-dg, and pFK-JFH1/H77/C842-dg to generate chimeric infectious HCV Con1/C3, J6/C3, and H77/C3, respectively, were given by R. Bartenschlager (University of Heidelberg, Heidelberg, Germany) [17]. Plasmids pH77C/JFH1, pJ4/JFH1, pJ6/JFH1, pJ8/JFH1, pS52/JFH1, pED43/JFH1, pSA13/JFH1, pHK6a/JFH1, and pQC69/JFH1 to generate chimeric infectious HCV were provided by J. Bukh (Copenhagen University Hospital, Hvidovre, Denmark) [18–22]. These chimeras are JFH1-based recombinants expressing core-NS2 of genotype 1 to 7 isolates. For the synthesis of HCV RNA, the plasmids were transcribed using a Megascript T7 kit (Ambion, Austin, TX, USA). To generate infectious HCV, the *in vitro* transcribed viral genomic RNA was transfected into

Huh7.5 cells by electroporation using a Gene Pulser system (Bio-Rad, Hercules, CA, USA) or by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. The culture supernatants collected at 2–7 days after transfection were centrifuged, passed through a 0.45 μm filter, and inoculated into naïve Huh7.5 cells. After additional passages on naïve cells, the cell-free supernatants containing HCV were concentrated approximately 10-fold using Amicon Ultra-15 (Millipore, Billerica, MA, USA) and measured for their infectivity titers. Aliquots were stored at −80°C until use.

Infectivity Titration

Virus titers were determined by focus-forming units (FFU) assay. Huh7.5 cells were seeded at 2×10^5 cells per well in 24-well plates and cultured overnight. Test samples were diluted serially 10-fold and each dilution was inoculated into the cells. After incubation for 6 h at 37°C, the cells were supplemented with fresh complete DMEM and cultured for 24 h. The cells were then immuno-stained and HCV-positive foci were manually counted under a fluorescence microscope. Each test was performed in duplicate or triplicate. The virus titer was expressed in FFU per ml sample, as determined by the mean number of IF-positive foci detected in a whole well.

Virus Neutralization Assays

Neutralization of HCV infection was assessed by the FFU reduction assay. Two independent assays were performed in the different laboratories. The first method was as follows: A 0.5 ml of serial 5-fold dilutions of #37, #55, or an irrelevant control antibody (human-IgG) (Sigma-Aldrich, St. Louis, MO, USA) was pre-incubated at 4°C overnight with an equal volume of the virus solution containing approximately 300 FFU/ml of HCV. The mixtures were inoculated into Huh7.5 cells (5×10^5/well) cultured on a 15mm-coverglass in 12-well plates. After incubation for 48 h, cells on the glass were fixed with cold 100% acetone and subjected to indirect IF for the detection of infected foci using a serum from patient H followed by the AlexaFluor 488 secondary antibody. IF-positive foci on the whole coverglass were manually counted under a fluorescence microscope. Each test was performed in duplicate. The second method was as follows: A 0.1 ml of the dilutions containing 0.2, 1, 3, 10, 30, 100, 300, or 1000 μg/ml of #55 or a control antibody was pre-incubated at 37°C for 1 h with an equal volume of the virus solution containing 10^2 or 10^3 FFU/0.1 ml of HCV. The mixtures were inoculated onto Huh7.5 cells (10^5 cells/well in 24-well plates). After 3 h of adsorption, the inocula were removed and fresh complete DMEM were added to the wells. At 24 h post-infection, cells were fixed with 4% paraformaldehyde (Wako, Tokyo, Japan) followed by permeabilization with 0.1% Triton-100 (Wako, Tokyo, Japan). The cells were then immuno-stained for the HCV proteins, counterstained with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA), and examined under a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). The number of HCV infected cells in each well was manually counted. The percent neutralization was calculated as the percent reduction of FFU compared with virus incubated with the control antibody. The NT_{50} value, lowest concentration (μg/ml) of antibody required for 50% reduction of FFU, was determined by curvilinear regression analysis.

Results

Establishment of Human Lymphoblastoid Cell Lines Producing Monoclonal Antibodies to the Envelope E2 Protein of HCV

PBMC obtained from patient H were infected with Epstein-Barr virus, strain B95-8, as we described previously [23], and cultured at 37°C in a 75 ml-flask in medium RPMI1640 (Life Technologies Japan, Tokyo, Japan) containing 10% FBS. Ten days later, the cells were distributed in 96-well plates in an amount of 10^4 cells/0.2 ml/well. After 4 days of cultivation, supernatant from each well was screened for presence of antibodies by IF using Huh 7 cells expressing the E2 protein (aa 384 to 661) derived from HCV strain H77. Cells in the well that gave a positive signal were re-distributed into 96-well plates and the wells were screened again. This procedure was repeated 5 times until all of the tested wells became positive on two successive assays. When cellular RNA was extracted and the V_H region of antibody was amplified by RT-PCR, identical sequence was obtained from three randomly selected wells, suggesting that the cells were clones. Antibody from this clone was designated as #37.

With a similar procedure we obtained antibody #55. For the screening of #55, Huh7 cells expressing the E2 protein derived from HCV strain H00 was employed as a target. **Figure 1** shows deduced amino acid sequences of the V_H regions for #37 and #55. The antibodies were isotyped by IF with Huh7 cells expressing the E2 protein (aa 384–661) of HCV strain H77, using specific secondary antibodies to human IgM, IgG1, IgG2, IgG3, and IgG4 subclasses, and to lambda and kappa light chains (Binding Site Inc., San Diego, CA, USA). As shown in **Figure 2**, #37 was IgG1/kappa and #55 was IgG1/lambda. The IgG was purified from the supernatants using a HiTrap protein G HP column (GE Healthcare, Uppsala, Sweden) and used for further characterization.

Epitope Mapping

Both #37 and #55 failed to react with the E2 protein in the western blot assay. Therefore, these antibodies were considered to recognize conformational epitopes. In order to map epitope sequences, we prepared expression vectors encoding various regions of the E2 protein derived from HCV strain H77. They include the regions aa 384 to 661, aa 411 to 661, aa 429 to 652, aa 429 to 607, aa 508 to 652, aa 429 to 552, aa 508 to 607, aa 552 to 652, aa 508 to 552, and aa 552 to 607. Huh7 cells expressing these regions were tested by IF for reactivity with #37 and #55. As shown in **Figure 3**, #37 was reactive with the expressed form of the E2 protein containing aa 429 to 652, but not with smaller sizes than this region. In contrast, #55 was reactive with the truncated form down to the region aa 508 to 607. These results indicate that the target epitopes of #37 and #55 are located in the regions of aa 429 to 652 and aa 508 to 607, respectively.

Cross-reactivity with Different HCV Genotypes and Binding Ability

Recent development of infectious chimeric HCV [17–22] has made it possible to investigate cross-genotype reactivity of the antibodies utilizing virus-infected cells as a target. We examined the cross-reactivity of #37 and #55 by IF using Huh7.5 cells infected HCV with different genotype E2 proteins. Genotypes tested were 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a, and 7a. Both #37 and #55 were reactive by IF with all genotypes tested.

Binding ability of #37 and #55 was assessed by measuring the minimum concentration of the antibodies required for an IF-

MAb	FR1	CDR1	FR2
#37	QVQLEQSGGGLVKPGESLRLSCAASGFILS	HYHMS	WFRQAPGKGLEWIA
#55	E----E------Q--G------E----MF-	AGW-H	-V--------V-VS

MAb	CDR2	FR3
#37	DINYSGRTTYEADSVRG	RFTVSRDNAKWSLYLQMNSLRVEDTAMYYCAR
#55	R---D-SS-TYV---K-	----------NT-F----------V----S

MAb	CDR3	FR4
#37	VGVVASINLMVGRRRSDNWFDL	WGQGTLVTVSS
#55	G-YYSYGPFGD.....	---------P-

Figure 1. Amino acid sequences of the V_H regions of #37 and #55. Residues identical to #37 sequences are indicated by a dash. Dots indicate gaps compared with the sequence of #37. MAb, monoclonal antibody; FR, framework regions; CDR, complementarity-determining regions.
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positive reaction using HCV-infected Huh7.5 cells as targets. The HCV inocula tested were strain JFH1 (genotype 2a) and ten chimeric HCV, including H77C/JFH1 (genotype 1a), J4/JFH1 (genotype 1b), Con1/C3 (genotype 1b), J6/JFH1 (genotype 2a), J8/JFH1 (genotype 2b), S52/JFH1 (genotype 3a), ED43/JFH1 (genotype 4a), SA13/JFH1 (genotype 5a), HK6a/JFH1 (genotype 6a) and QC69/JFH1 (genotype 7a). Antibody solutions containing 5 μg/ml of IgG were two-fold serially-diluted and each dilution was tested by IF for a positive reaction. The results are shown in **Table 1**. The minimum concentration of #55 required was 10–78 ng/ml, while that of #37 was 156–1250 ng/ml except for H77C/JFH1 and S52/JFH1, which required 20 ng/ml. The higher ability of #37 in binding to H77C/JFH1 was possibly because this chimeric virus was a JFH1-based recombinant with homologous envelope E2 of strain H77 from the PBMC-donor. The higher reactivity with S52/JFH1 remains unexplained. Overall, more of #37 was needed for a positive reaction compared to #55, indicating that #55 has a higher binding ability than #37.

Distribution of Reacting Antigens in the HCV-infected Cells

Distribution of the antigens reacting with #55 and #37 in the HCV-infected cells was observed by IF-staining. We

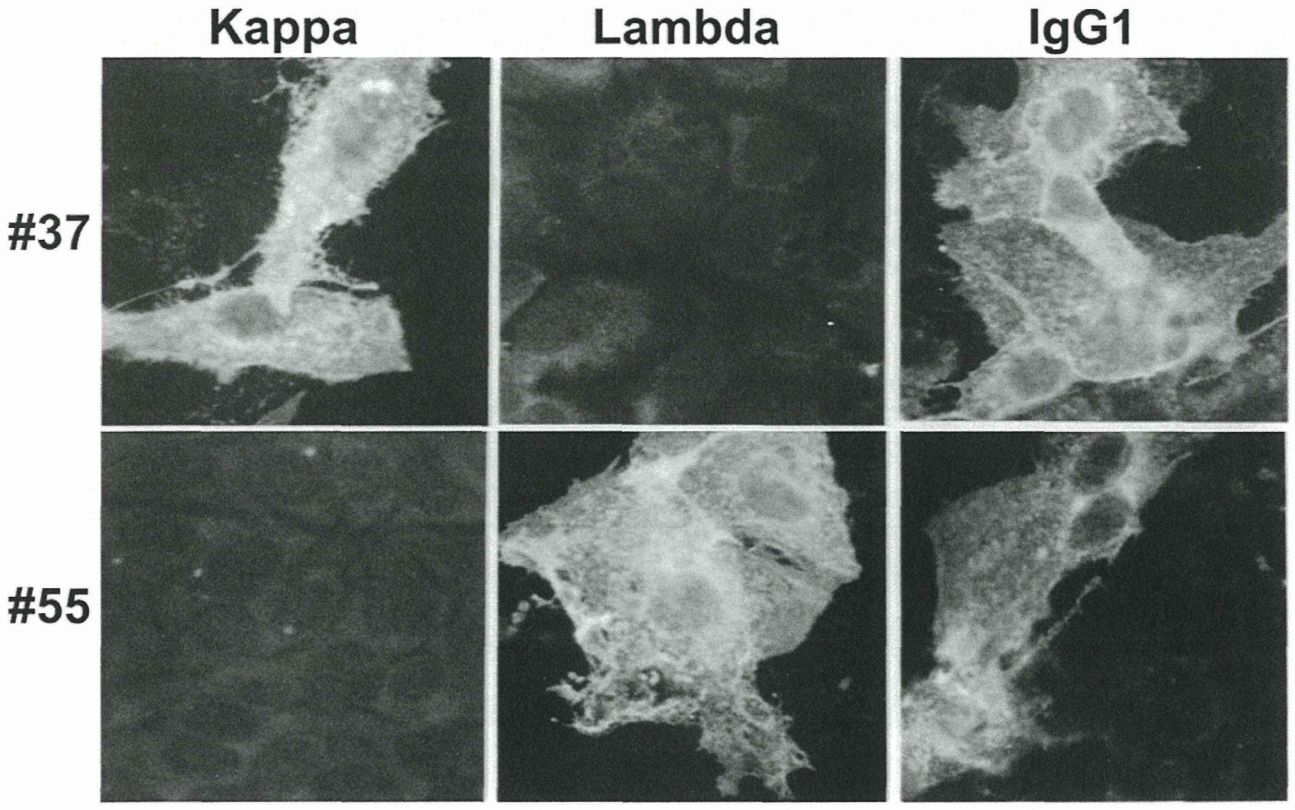

Figure 2. Isotyping revealed that #37 and #55 were IgG1/kappa and IgG1/lambda, respectively. Huh7 cells expressing the HCV E2 (aa 384–661) derived from strain H77 were incubated with #37 or #55, washed, and stained with fluoresceinated anti-human IgG1, anti-human lambda, or anti-human kappa.
doi:10.1371/journal.pone.0055874.g002

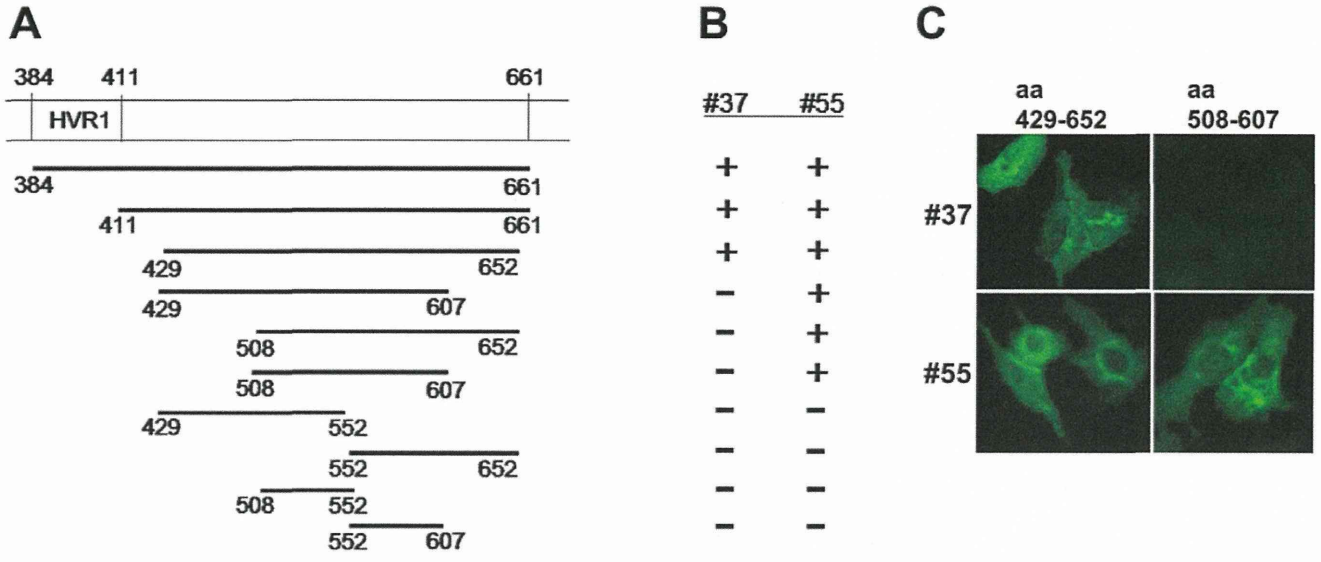

Figure 3. Epitope mapping revealed that #37 and #55 recognized the regions aa 429 to 652 and aa 508 to 607 of the E2 protein, respectively. (**A**). A panel of Huh7 cells expressing various truncations of the E2 protein derived from HCV strain H77 was generated. At the top of the graphic, aa 384 to 661 of the E2 protein is depicted with aa numbers. HVR1, hyper variable region 1. (**B**). Results of the assays for reactivity by IF. +, positive recognition; -, negative recognition. (**C**). IF-reactions of #37 and #55 against the cells expressing aa 429–652 and aa 508–607.
doi:10.1371/journal.pone.0055874.g003

examined Huh7.5 cells infected with 12 different inocula of HCV, including strain JFH1 and eleven chimeric HCV with structural proteins derived from various genotypes. **Figure 4** shows IF positive-staining by #37 and #55 observed in the cells infected with strain JFH1. The antibodies produced a different pattern of staining; while #37 gave coarse granular staining mostly located in the periphery of the nucleus, #55 gave diffuse staining throughout the cytoplasm. Similar patterns of IF-staining were observed for other chimeric HCV tested, including H77C/JFH1, J4/JFH1, J6/JFH1, J8/JFH1, S52/JFH1, ED43/JFH1, SA13/JFH1, HK6a/JFH1, QC69/JFH1, H77/C3, and Con1/C3. An irrelevant control antibody (human IgG) did not give such positive staining in the infected cells. #37, #55, and the control antibody were not reactive with non-infected Huh7.5 cells.

Ability to Recognize HCV Particles

It was recently reported that cell culture-grown HCV particles were pleomorphic, 40–75 nm in diameter, and spherical [24]. To determine whether #37 and #55 are able to recognize intact viral particles, we performed indirect immuno-gold EM using anti-human IgG labeled with colloidal gold particles as a second antibody. As target HCV for this experiment, we employed H77/C3 with homologous envelope E2 of strain H77 from the PBMC-donor. Concentration and purification of viral particles from culture supernatants was carried out by equilibrium SDG centrifugation. **Figure 5A** shows the distribution of HCV RNA measured by RT-qPCR after the centrifugation. Two peaks of viral RNA were obtained at 1.076 g/ml and 1.171 g/ml in fractions 3 and 6, respectively. Copy numbers of HCV RNA were 4.3×10^{5}/0.1 ml for fraction 3 and 7.4×10^{5}/0.1 ml for fraction 6.

Table 1. Binding activity measured by immunofluorescence.

Virus (genotype)	Minimum concentration (ng/ml) required for positive reaction	
	#37	#55
H77C/JFH1 (1a)	20	10
J4/JFH1 (1b)	156	78
J6/JFH1 (2a)	1250	20
J8/JFH1 (2b)	1250	78
S52/JFH1 (3a)	20	10
ED43/JFH1 (4a)	625	20
SA13/JFH1 (5a)	156	78
HK6a/JFH1 (6a)	156	78
QC69/JFH1 (7a)	1250	78
Con1/C3 (1b)	625	78
JFH1 (2a)	313	78

doi:10.1371/journal.pone.0055874.t001

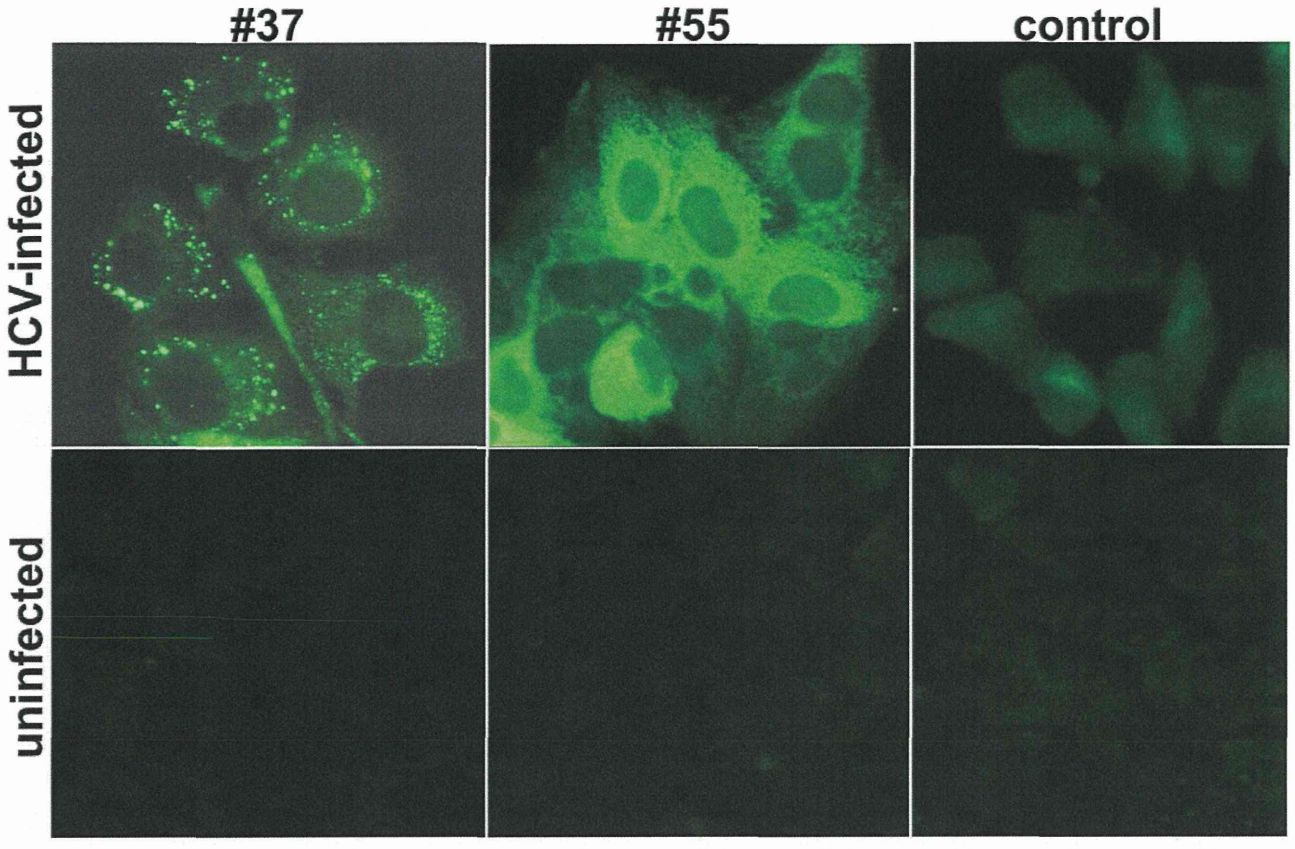

Figure 4. Different pattern of IF-staining by #37 and #55 in the HCV-infected Huh7.5 cells. #37 gave granular IF-reactions scattered in the cytoplasm. #55 gave diffuse staining throughout the cytoplasm. The control antibody (human IgG) gave negative staining. #37, #55, and the control antibody were not reactive with uninfected Huh7.5 cells.
doi:10.1371/journal.pone.0055874.g004

These two fractions were further examined by the FFU assay for their infectivity titers. Fractions 3 and 6 had an infectivity titer of 2.0×10^4 FFU/0.1 ml and 4.7×10^3 FFU/0.1 ml, respectively. Fraction 3 was calculated to have an approximately 9 times higher infectivity titer per HCV RNA than fraction 6 (**Figure 5B**), which was in accordance with our previous observation that the fraction with lower buoyant density was more infectious [25]. Thus, we selected fraction 3 for the EM examination. Fraction 3 was treated with #37 followed by anti-human IgG labeled with colloidal gold-particles, negatively stained, and examined in a transmission electron microscope. We detected HCV-like particles coated with colloidal gold, indicating the binding of #37 to virions. Most of the viral particles reacting with #37 measured approximately 50–60 nm in diameter. **Figure 5C (a)** shows an aggregate of three virions coated with specific gold. These viral particles measured approximately 50 nm in diameter. **Figure 5C (b)** shows two particles; the one on the right (50-nm in diameter) was coated with colloidal gold, indicating the binding of #37. Another particle on the left (35-nm in diameter) was negative for colloidal gold, indicating that #37 was not reactive with this particle. In addition, the presence of such uncoated particle in the same field suggested that colloidal gold did not bind non-specifically. When fraction 3 was reacted with #55 followed by anti-human IgG labeled with colloidal gold particles, larger aggregates of various-sized viral particles were observed, as shown in **Figure 5C (c)**. The viral particles varied in sizes from 40 to 70 nm in diameter. Immuno-gold EM demonstrated that both #37 and #55 can bind to HCV particles. **Figure 5C (d)** shows negative reaction of colloidal gold by an irrelevant control antibody (human IgG).

Neutralizing Activity

To investigate whether #37 and #55 could inhibit HCV infection, we performed an *in vitro* neutralization assay by reduction of FFU. As HCV inocula, we used chimeric H77/C3 (genotype 1a), chimeric Con1/C3 (genotype 1b), and chimeric J6/C3 (genotype 2a) in this assay. A virus sample containing approximately 300 FFU/ml of HCV was pretreated at 4°C overnight with #37, #55, or an irrelevant control antibody at a final concentration of 0.1, 0.5, 2.5, 12.5, 62.5, or 312.5 μg/ml and the mixtures were then inoculated into Huh7.5 cells. After 48 h post-infection, IF-positive foci were manually counted under a fluorescence microscope. Each test was performed in duplicate. As shown in **Figure 6**, compared to the results obtained with an irrelevant control antibody, #55 inhibited the viral infection in dose-dependent manner for all of the 3 samples tested. Inhibition by #37 was not observed.

Since #55 was found to have a neutralizing activity as shown above, further examination by the FFU reduction assay was conducted with HCV strain JFH1 (genotype 2) and various chimeric HCV containing the E2 proteins from 9 different genotypes. Chimeric viruses tested included H77C/JFH1 (genotype 1a), J4/JFH1 (genotype 1b), J6/JFH1 (genotype 2a), J8/JFH1 (genotype 2b), S52/JFH1 (genotype 3a), ED43/JFH1 (genotype 4a), SA13/JFH1 (genotype 5a), HK6a/JFH1 (genotype 6a), and QC69/JFH1 (genotype 7a). Two different concentrations of target

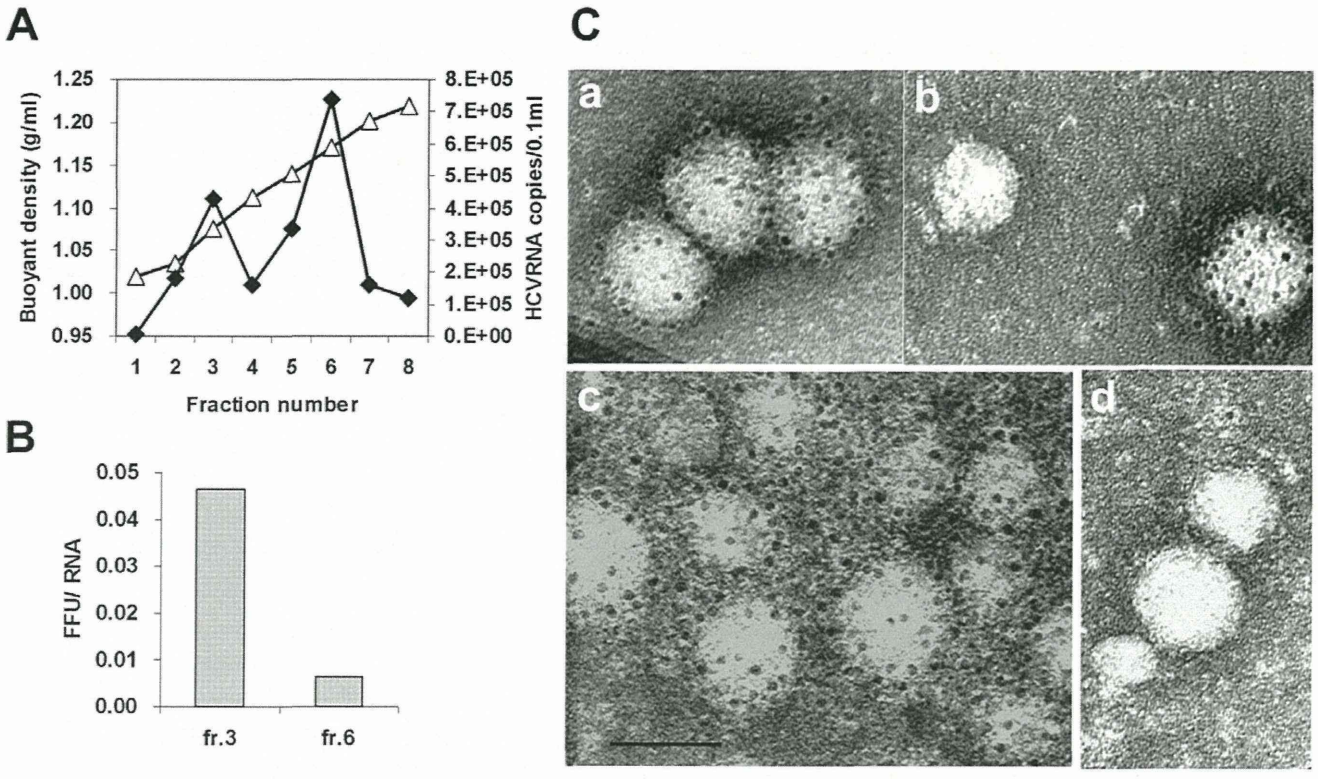

Figure 5. Immuno-gold EM demonstrated that both #37 and #55 recognized HCV intact particles. (**A**). Equilibrium SDG centrifugation. ◆, HCV RNA copies measured by RT-qPCR; Δ, buoyant densities. (**B**). The ratio of infectivity titer (FFU) to HCV RNA copies of fractions 3 and 6. (**C**). Immuno-gold EM. Viral particles in fraction 3 were treated with #37 (**a, b**), #55 (**c**), or a control antibody (**d**) followed by anti-human IgG conjugated with colloidal gold particles, and examined under an electron microscope. HCV-like particles in the sample treated with #37 and #55 were observed with specific labeling of gold particles indicating that the antibodies are capable of binding to viral particles. Bar = 50 nm.
doi:10.1371/journal.pone.0055874.g005

HCV were tested in the assays. The one virus-sample contained 10^2 FFU/0.1 ml (no.1) and another 10^3 FFU/0.1 ml (no.2). **Table 2** shows the 50% neutralization titers (NT_{50}) of #55, a lowest concentration (μg/ml) required for 50% reduction of FFU, calculated by curvilinear regression analysis. #55 neutralized HCV infection of various genotypes (1a, 1b, 2a, 3a, 4a, 5a, and 6a), with the NT_{50} titers ranging from 2 to 127 μg/ml for no.1 and 6 to 231 μg/ml for no.2. Neutralization of genotype 7a (QC69/JFH1) by #55 was less, with a NT_{50} titer of 219 μg/ml for no.1 and >500 μg/ml for no.2.

Blocking of Viral Adsorption

We examined whether #55 blocked viral adsorption to cells by measuring the amount of cell-attached HCV RNA using RT-qPCR. A half ml of the culture supernatant containing 10^5 FFU/ml of chimeric HCV, H77/C3, was pre-treated at 4°C for 24 h with an equal volume of #55, #37 or a control antibody at a final

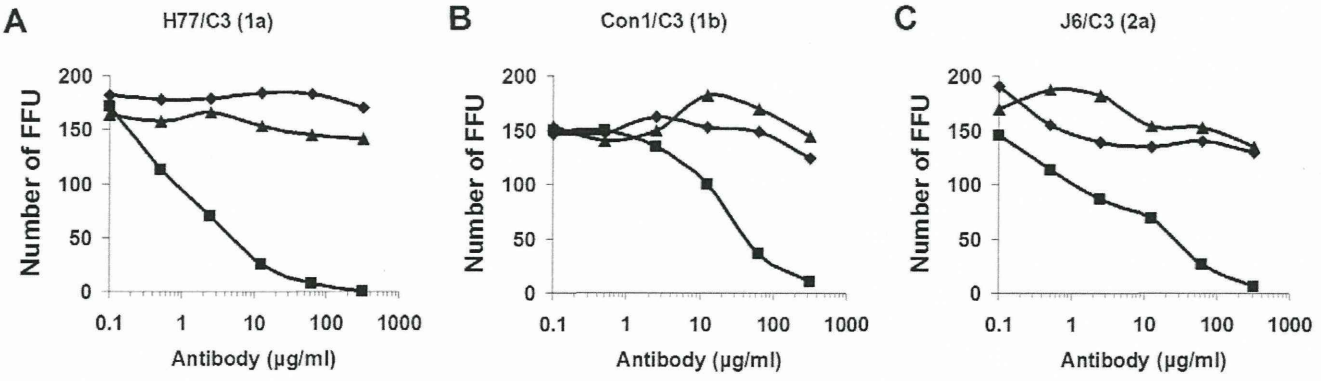

Figure 6. Neutralization assay by FFU reduction. The mean numbers of positive foci are shown for viruses H77/C3 (**A**), Con1/C3 (**B**), and J6/C3 (**C**). Compared to the results obtained with an irrelevant control antibody, #55 inhibited the viral infection in dose-dependent manner for all of the 3 samples tested. Inhibition by #37 was not observed. ◆, control; ■, #55; ▲, #37.
doi:10.1371/journal.pone.0055874.g006

Table 2. 50% neutralization titers (NT_{50}) of #55 by FFU reduction.

Virus (genotype)	NT_{50} (μg/ml)*	
	no.1	no.2
H77C/JFH1 (1a)	10.6	10.3
J4/JFH1 (1b)	ND	9.3
J6/JFH1 (2a)	126.6	99.1
J8/JFH1 (2b)	21.1	24.1
S52/JFH1 (3a)	73.0	230.8
ED43/JFH1 (4a)	1.3	7.4
SA13/JFH1 (5a)	5.0	5.7
HK6a/JFH1 (6a)	5.2	7.3
QC69/JFH1 (7a)	218.5	>500
JFH1 (2a)	2.0	ND

*, calculated by curvilinear regression analysis; ND, not done.
doi:10.1371/journal.pone.0055874.t002

concentration of 500, 50, or 5 μg/ml. The mixtures were then inoculated onto Huh7.5 cells seeded in 12-well plates (5×10^5 cells/well). After incubation for 4 h at 37°C, cells were washed 3 times with PBS. Amount of cell-associated HCV RNA in a well was measured by RT-qPCR. Each test was performed in duplicate. Compared to the control antibody (human IgG), #55 inhibited viral adsorption in dose-dependent manner, as shown in **Figure 7**. Inhibition by #37 was not observed but rather slightly enhanced at a concentration of 50 μg/ml.

Specific amino acids (W420, Y527, W529, G530, and D535) in the E2 envelope protein of HCV were reported to be critical for binding to CD81, a principal cellular receptor and they were conserved across all genotypes [26]. As the epitope of #55 includes these amino acid residues, it was possible that #55 blocked virus adsorption by competing with CD81 for a binding site on the E2 envelope. **Figure 8A** shows sequence alignment of aa 508 to 607, the epitope of #55, of HCV employed in the present study. The epitope of #55 contains the residues important for binding to CD81 (asterisks). Thus, we investigated this possibility by testing whether CD81 inhibits binding of HCV to #55 utilizing an assay based on antibody-captured RT-qPCR. A

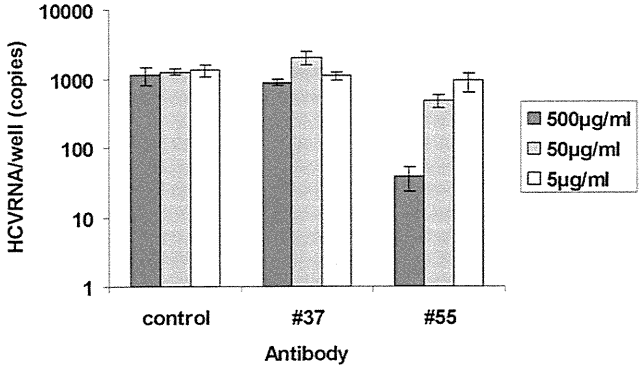

Figure 7. Blocking of viral adsorption by #55. Blocking of viral adsorption measured by RT-qPCR. Compared to the control antibody (human IgG), #55 inhibited viral adsorption in dose-dependent manner. Inhibition by #37 was not observed but rather slightly enhanced at a concentration of 50 μg/ml.
doi:10.1371/journal.pone.0055874.g007

100 μl of the virus solution containing 10^4 FFU/ml of H77/C3 was incubated with an equal volume of various dilutions of soluble recombinant human CD81 protein (Origene, Rockville, MD, USA) for 2 h at room temperature. Each mixture was then inoculated into a 48-well plate which was pre-coated with #55 or an irrelevant control antibody (human IgG) at a concentration of 10 μg/ml. The plate was incubated at 4°C overnight. After washing, bound HCV RNA was extracted and quantified by RT-qPCR. As shown in **Figure 8B**, CD81 inhibited the binding of virus to #55 in a dose-dependent manner.

Discussion

In this study, as an approach to obtain human B cell lines producing antibodies to HCV envelope E2, we applied the EBV transformation method, which is based on the fact that EBV transforms B-lymphocytes of humans *in vitro* into lymphoblastoid cells that synthesize and secrete immunoglobulins. From PBMC collected from a patient persistently infected with HCV strain H (genotype 1a) we have successfully isolated two clones producing anti-HCV E2 antibodies, #37 and #55. At the first screening of culture supernatants, several wells of a 96-well plate were found positive for anti-HCV E2 antibodies. However, most of them became negative as further cultured. Finally #37 and #55 remained as stably producing clones.

There was remarkable contrast between these two antibodies in their properties: (1) #55 appeared to be a broadly cross-neutralizing antibody. In the neutralization assay by FFU reduction, it inhibited infection by HCV genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a, and, to a lesser extent, 7a. In contrast, #37 did not neutralize any of the viruses tested. Interestingly it tended to enhance the infection at low concentrations (**Figure 6B and C,** and **Figure 7**): (2) the epitope of #55 was mapped to the region of aa 508 to 607 and that of #37 was mapped to the longer region spanning aa 429 to 652 of the E2 protein. #55 seemed unique for broadly cross-neutralizing antibody to have a relatively short conformational epitope, since it has been reported that conformational epitopes reacting with such antibodies are usually retained in the full length E2 [7,9]: (3) when we tested their cross-reactivity using transfected Huh7 cells expressing the E2 proteins, #37 was reactive with genotype 1a but reacted very weakly with the others, while #55 was broadly reactive with all genotypes tested. However, when examined using the virus-infected cells as targets, #37 was reactive with all HCV genotypes tested, although its binding activity measured by IF was less than that of #55 except for H77C/JFH1(1a) and S52/JFH(3a): (4) in immuno-gold EM, viral particles recognized by #37 were rather homogenous in size and measured approximately 50–60 nm in diameter. On the other hand, #55 produced larger aggregates of various-sized viral particles, probably because of its higher binding activity: (5) the antibodies showed a different pattern of IF-staining in the HCV-infected cells. While #37 gave granular reactions mostly in the periphery of nuclei, #55 gave diffuse staining throughout the cytoplasm (**Figure 4**). The nature of the antigens reacting with #37 and #55 remains to be studied.

Recently, Keck et al. reported that the region aa 529 to 535 of the E2 envelope protein is a CD81 binding region that does not tolerate neutralization escape mutations [27]. The epitope of #55 includes the above mentioned region and #55 blocked virus adsorption by competing with CD 81 for a binding site on the E2 envelope. As #55 is broadly neutralization cross-reactive, it may be very useful in preventing infection by HCV of various genotypes. Sasayama et al. reported that blocking N-glycosylation of aa 534 (aa 532 of strain H77) in this region by substituting

A

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                   508             • • • ••   •             552                                                  607  References
H77/C3(1a)         CFTPSPVVVGTTDRSGAPTYSWGANDTDVFVLNNTRPPLGNWFGCTWMNSTGFTKVCGAPPCVI.GGVGNNT....LLCPTDCFPKHPEATYSRCGSGPWITPRC  [17]
Con1/C3(1b)        --------------F-V------E-E---LL-------Q----------------T--G---N-.--I--K-....-T------R-------TK------L----  [17]
J6/C3(2a)          --------------L-----T--E-E----L--S------S--------------T------RT.RADFNASTD..--------R---DT--LK------L----  [17]
JFH1(2a)           --------------R-V---T--E-E----L--S----Q-S---------S-Y--T------RT.RADFNASTD..--------R---D---IK------L--K-  [5]
H77C/JFH1(1a)      ----------------------------------------------------------------.-------....--------R--------------------  [20]
J4/JFH1(1b)        ----------------V------E-E---ML-------Q----------------T--G---N-.-----R-....-I------R-------TK------L----  [18]
J6/JFH1(2a)        --------------L-----T--E-E----L--S------S---------S-Y--T------RT.RADFNASTD..--------R---DT--LK------L----  [19]
J8/JFH1(2b)        -------------KQ-V---T--E-E----L--S----R-A--------G-----T------R-.RKDY-S-ID..--------R---D---LK--A---L----  [18]
S52/JFH1(3a)       -------------IK-K---N--E-E----L-ESL---S-R----A-------L-T------N-Y--E-DPENETD-F------R-----------A---L----  [22]
ED43/JFH1(4a)      -------------HV-V---T--E-E----L--S----H-A----V---------T------EV.NTNNGT.....WH------R----T--AK----------  [20]
SA13/JFH1(5a)      --------------K-N------E-E--I-L-------T--------------V-T------NL.-PT---S.....K------R---D---TK------L----  [21]
HK6a/JFH1(6a)      -------------KL-I---N--E-E----M-ESL---T-G--------------T------Q-.VPGDY-SSANE--------R-------Q------------  [18]
QC69/JFH1(7a)      --------------R-V---T--E-ES---L--S----Q-S----S---T-----T--G---K-.RPQ-AQSNTS.-T------R---R----A------L----  [18]
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☆, important amino acids for binding to CD81[26]

B

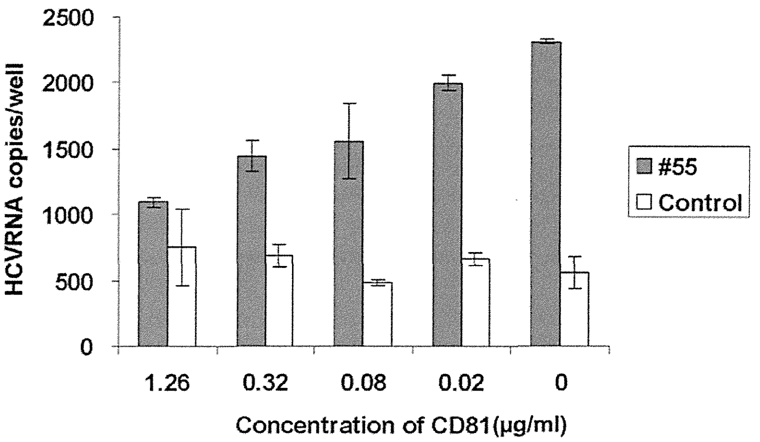

Figure 8. Binding of HCV to #55 was inhibited by soluble recombinant CD81. (**A**). Sequence alignment of aa 508 to 607, the epitope of #55, of various genotypes of HCV employed in the present study. Residues identical to the sequences of H77/C3 are indicated by a dash. Dots indicate gaps. (**B**). H77/C3 virus was treated with various dilutions of soluble CD81.The mixtures were inoculated into a 48-well plate that was pre-coated with #55 or the control antibody. Amount of bound HCV RNA was measured by RT-qPCR.
doi:10.1371/journal.pone.0055874.g008

asparagine with histidine markedly enhanced the sensitivity of the virus to neutralizing antibodies and suggested that the aa 529 to 535 region is usually protected from the antibody's access by the N-glycosylation [28]. It is possible that #55 may evade the N-glycosylation mediated protective mechanism of HCV.

A cross-neutralizing monoclonal antibody that could be generated in large volume might be particularly beneficial to prevent the almost universal occurrence of HCV re-infection of transplanted livers and could play other roles in immunoprophylaxis until such time as an effective HCV vaccine is developed and commercialized.

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Author Contributions

Conceived and designed the experiments: YKS RHP. Performed the experiments: YKS MH MO. Analyzed the data: YKS KS HY HH. Contributed reagents/materials/analysis tools: HJA. Wrote the paper: YKS RHP HJA HY.

References

1. Shimizu YK, Hijikata M, Iwamoto A, Alter HJ, Purcell RH, et al. (1994) Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. J Virol 68: 1494–1500.
2. Logvinoff C, Major ME, Oldash D, Heyward S, Talal A, et al. (2004) Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proc Natl Acad Sci U S A 101: 10149–10154.
3. Ishii K, Rosa D, Watanabe Y, Katayama T, Harada H, et al. (1998) High titers of antibodies inhibiting the binding of envelope to human cells correlate with natural resolution of chronic hepatitis C. Hepatology 28: 1117–1120.
4. Farci P, Alter HJ, Wong DC, Miller RH, Govindarajan S, et al. (1996) Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. Proc Natl Acad Sci U S A 93: 15394–15399.
5. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, et al. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11: 791–796.
6. Meunier JC, Russell RS, Goossens V, Priem S, Walter H, et al. (2008) Isolation and characterization of broadly neutralizing human monoclonal antibodies to the E1 glycoprotein of hepatitis C virus. J Virol 82: 966–973.
7. Perotti M, Mancini N, Diotti RA, Tarr AW, Ball JK, et al. (2008) Identification of a broadly cross reacting and neutralizing human monoclonal antibody directed against the hepatitis C virus E2 protein. J Virol 82: 1047–1052.
8. Owsianka AM, Tarr AW, Keck ZY, Li TK, Witteveldt J, et al. (2008) Broadly neutralizing human monoclonal antibodies to the hepatitis C virus E2 glycoprotein. J Gen Virol 89: 653–659.

9. Mancini N, Diotti BA, Perotti M, Sautto G, Clementi N, et al. (2009) Hepatitis C virus (HCV) infection may elicit neutralizing antibodies targeting epitopes conserved in all viral genotypes. PLoS ONS 4: 1–7.
10. Broering TJ, Garrity KA, Boatright NK, Sloan SE, Sandor F, et al. (2009) Identification and Characterization of broadly neutralizing human monoclonal antibodies directed against the E2 envelope glycoprotein of hepatitis C virus. J Virol 83: 12473–12482.
11. Giang E, Dorner M, Prentoe JC, Dreux M, Evans MJ, et al. (2012) Human broadly neutralizing antibodies to the envelope glycoprotein complex of hepatitis C virus. Proc Natl Acad Sci U S A 109: 6205–6210.
12. Feinstone SM, Alter HJ, Dienes P, Shimizu Y, Popper H, et al. (1981) Non-A, non-B hepatitis in chimpanzees and marmosets. J Infect Dis 144: 588–597.
13. Blight KJ, Mckeating JA, Rice CM (2002) Highly permissive cell lines for subgenomic and genomic hepatitis C virus. J Virol 76: 13001–13014.
14. Shimizu YK, Purcell RH, Yoshikura H (1993) Correlation between the infectivity of hepatitis C virus in vivo and its infectivity in vitro. Proc Natl Acad Sci U S A 90: 6037–6041.
15. Shimizu YK, Hijikata M, Oshima M, Shimizu K, Yoshikura H (2006) Detection of a 5′ end subgenome of hepatitis C virus terminating at nucleotide 384 in patients' plasma and liver tissues. J Viral Hepatitis 13: 746–755.
16. Forns X, Allander T, Rohwer-Nutter P, Bukh J (2000) Characterization of modified hepatitis C virus E2 proteins expressed on the cell surface. Virology 274: 75–85.
17. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis AS, et al. (2006) Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C chimeras. Proc Natl Acad Sci U S A 103: 7408–7413.
18. Gottwein JM, Scheel TK, Jensen TB, Lademann JB, Prentoe JC, et al. (2009) Development and characterization of hepatitis C virus genotype 1–7 cell culture systems: role of CD81 and Scavenger Receptor Class B type 1 and effect of antiviral drugs. Hepatology 49: 364–377.
19. Yi-Ping L, Gottwein JM, Scheel TK, Jensen TB, Bukh J (2011) MicroRNA-122 antagonism against hepatitis C virus genotypes1–6 and reduced efficacy by host RNA insertion or mutations in the HCV 5′ UTR. Proc Natl Acad Sci U S A 108: 4991–4996.
20. Scheel TK, Gottwein JM, Jensen TB, Prentoe JC, Hoegh AM, et al. (2008) Development of JFH1-based cell culture systems for hepatitis C virus genotype 4a and evidence for cross-genotype neutralization. Proc Natl Acad Sci U S A 105: 997–1002.
21. Jensen TB, Gottwein JM, Scheel TK, Hoegh AM, Eugen-Olsen J, et al. (2008) Highly efficient JFH1-based cell culture system for hepatitis C virus genotype 5a: Failure of homologous neutralization-antibody treatment to control infection. J Infect Dis 198: 1756–1765.
22. Gottwein JM, Scheel TK, Hoegh AM, Lademann JB, Eugen-Olsen J, et al. (2007) Robust hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses. Gastroenterology 133: 1614–1624.
23. Shimizu YK, Oomura M, Abe K, Uno M, Yamada E, et al. (1985) Production of antibody associated with non-A, non-B hepatitis in a chimpanzee lymphoblastoid cell line established by in vitro transformation with Epstein-Barr virus. Proc Natl Acad Sci U S A 82: 2138–2142.
24. Gastaminza P, Dryden K, Boyd B, Wood M, Law M, et al. (2010) Ultrastructural and biophysical characterization of hepatitis C virus particles produced in cell culture. J Virol 84: 10999–11009.
25. Hijikata M, Shimizu YK, Kato H, Iwamoto A, Shih JW, et al. (1993) Equilibrium centrifugation studies of hepatitis C virus: Evidence for circulating immune complexes. J Virol 67: 1953–1958.
26. Owsianka AM, Timms JM, Tarr AW, Brown JP, Hickling TP, et al. (2006) Identification of conserved residues in the E2 glycoprotein of the hepatitis C virus that are critical for CD81 binding. J Virol 80: 8695–8704.
27. Keck ZY, Saha A, Xia J, Wang Y, Lau P, et al. (2011) Mapping a region of hepatitis C virus E2 that is responsible for escape from neutralizing antibodies and a core CD81-binding region that does not tolerate neutralization escape mutations. J Virol 85: 10451–10463.
28. Sasayama M, Shoji I, Adianti M, Jiang DP, Deng L, et al. (2012) A point mutation at asn-534 that disrupts a conserved N-glycosylation motif of the E2 glycoprotein of hepatitis C virus markedly enhances the sensitivity to antibody neutralization. J Med Virol 84: 229–234.

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Visualization and Measurement of ATP Levels in Living Cells Replicating Hepatitis C Virus Genome RNA

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Abstract

Adenosine 5′-triphosphate (ATP) is the primary energy currency of all living organisms and participates in a variety of cellular processes. Although ATP requirements during viral lifecycles have been examined in a number of studies, a method by which ATP production can be monitored in real-time, and by which ATP can be quantified in individual cells and subcellular compartments, is lacking, thereby hindering studies aimed at elucidating the precise mechanisms by which viral replication energized by ATP is controlled. In this study, we investigated the fluctuation and distribution of ATP in cells during RNA replication of the hepatitis C virus (HCV), a member of the *Flaviviridae* family. We demonstrated that cells involved in viral RNA replication actively consumed ATP, thereby reducing cytoplasmic ATP levels. Subsequently, a method to measure ATP levels at putative subcellular sites of HCV RNA replication in living cells was developed by introducing a recently-established Förster resonance energy transfer (FRET)-based ATP indicator, called ATeam, into the NS5A coding region of the HCV replicon. Using this method, we were able to observe the formation of ATP-enriched dot-like structures, which co-localize with non-structural viral proteins, within the cytoplasm of HCV-replicating cells but not in non-replicating cells. The obtained FRET signals allowed us to estimate ATP concentrations within HCV replicating cells as ~5 mM at possible replicating sites and ~1 mM at peripheral sites that did not appear to be involved in HCV replication. In contrast, cytoplasmic ATP levels in non-replicating Huh-7 cells were estimated as ~2 mM. To our knowledge, this is the first study to demonstrate changes in ATP concentration within cells during replication of the HCV genome and increased ATP levels at distinct sites within replicating cells. ATeam may be a powerful tool for the study of energy metabolism during replication of the viral genome.

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Introduction

Adenosine 5′-triphosphate (ATP) is the major energy currency of cells and is involved in a variety of cellular processes, including the virus life cycle, in which ATP-dependent reactions essential for virus multiplication are catalyzed by viral-encoded enzymes or complexes consisting of viral and host-cell proteins [1]. However, the lack of a real-time monitoring system for ATP has hindered studies aimed at elucidating the mechanisms by which cellular processes are controlled through ATP. A method for measuring ATP levels in individual living cells has recently been developed using a genetically-encoded FRET-based indicator for ATP, called ATeam, which employs the epsilon subunit of a bacterial F_0F_1-ATPase [2]. The epsilon subunit has several theoretical advantages for use as an ATP indicator; i) small size (14 kDa), ii) high specific binding to ATP, iii) ATP binding induces a global conformational change and iv) ATP hydrolysis does not occur following binding [3–5]. The affinity of ATeam for ATP can be adjusted by changing various amino acid residues in the ATP-binding domain within the subunit. ATeam has enabled researchers to examine the subcellular compartmentation of ATP as well as time-dependent changes in cellular ATP levels under various physiological conditions. For example, the ATeam-based method has been used to demonstrate that ATP levels within the mitochondrial matrix are lower than those in the cytoplasm and the nucleus [2].

Hepatitis C virus (HCV) infects 2–3% of the world population and is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [6–8]. HCV possesses a positive-strand RNA genome and belongs to the family *Flaviviridae*. A precursor polyprotein of ~3000 amino acids is post- or co-translationally processed by both viral and host proteases into at least ten viral products. The nonstructural (NS) proteins NS3, NS4A, NS4B, NS5A and NS5B are necessary and sufficient for autonomous HCV RNA replication. These proteins form a membrane-associated replication complex (RC), in which NS5B is the RNA-dependent RNA polymerase (RdRp) responsible for copying the RNA genome of the virus during replication [9,10]. NS3, in addition to its protease activity, functions as a viral helicase capable of separating duplex RNA and DNA in reactions fuelled