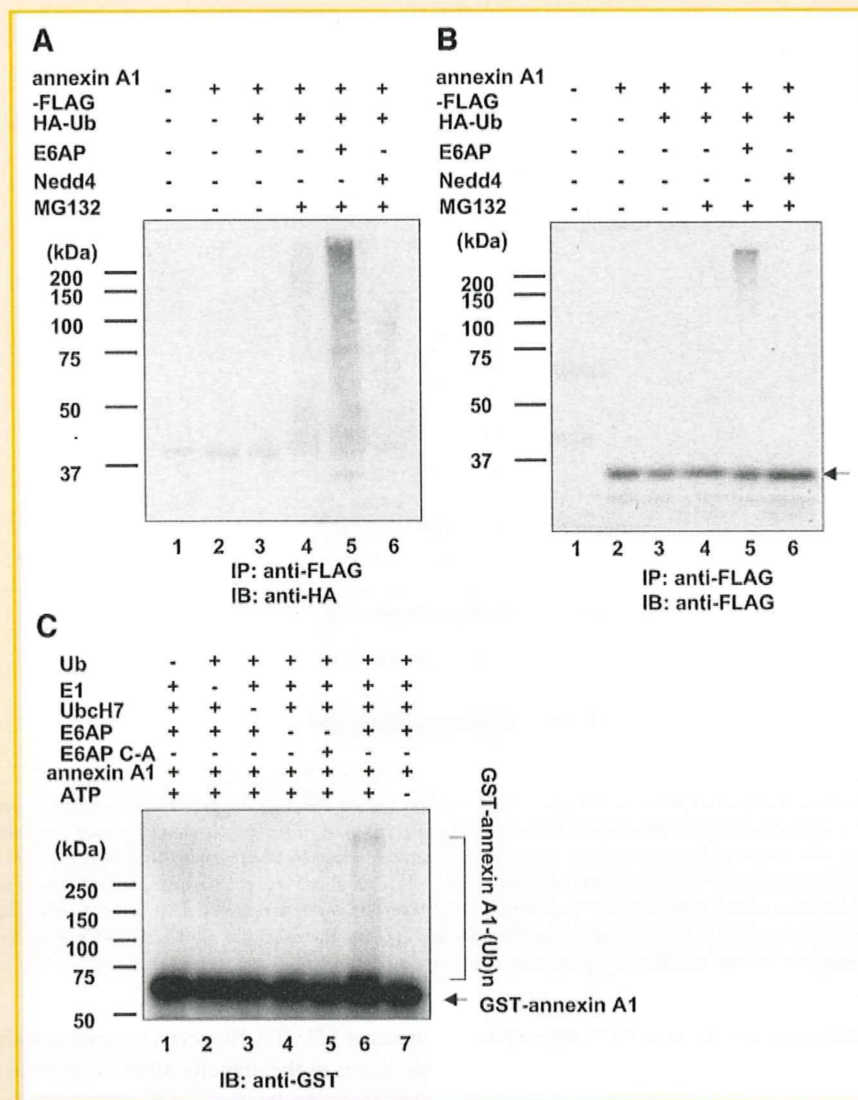

Fig. 5. E6AP-dependent ubiquitylation of annexin A1 protein in vivo and in vitro. HEK 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG annexin-FLAG together with 2 μg of plasmid encoding E6AP as indicated. Each transfection also included 2 μg of plasmid encoding HA-ubiquitin. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb (A) or anti-FLAG PAb (B). The Western blot shows the presence of a ubiquitin smear. The arrow indicates annexin-FLAG. IB, immunoblot; IP, immunoprecipitation. C: In vitro ubiquitylation of annexin A1 by E6AP. For in vitro ubiquitylation of annexin A1 protein, purified GST-annexin A1 was used as a substrate. Assays were done in 40-μl volumes containing each component as indicated. The reaction mixture is described in the Experimental Procedures. The reaction mixture contained 1 mM $CaCl_2$. The reaction was terminated by addition of SDS-PAGE loading buffer and followed by immunoblotting with anti-GST PAb. The arrow indicates GST-annexin A1. Ubiquitylated species of GST-annexin A1 proteins are marked by brackets.

DISCUSSION

In the present study, we have identified annexin A1 as a novel substrate for E6AP using four lines of evidence: (1) E6AP bound to annexin A1 in vivo and in vitro; (2) overexpression of E6AP enhanced proteasomal degradation of annexin A1 in vivo; (3) knockdown of endogenous E6AP by siRNA resulted in the accumulation of endogenous annexin A1 in vivo; and (4) E6AP enhanced the polyubiquitylation of annexin A1 in vivo and in vitro. These results provide evidence that E6AP mediates the ubiquitylation and proteasomal degradation of annexin A1. We have shown that E6AP bound to annexin A1 only in the presence of Ca^{2+} and that these interactions were enhanced by increasing concentrations of Ca^{2+}. Annexin A1 was polyubiquitylated by E6AP only in the presence of Ca^{2+}. Chelating Ca^{2+} with EGTA inhibited E6AP-mediated polyubiquitylation of annexin A1. The E6AP-binding domain on annexin A1 was mapped to the core domain, especially the annexin repeat domain III. These results suggest that the conformational change of annexin A1 induced by Ca^{2+} binding allows E6AP to bind to annexin repeat domain III of annexin A1 and to mediate its ubiquitylation and degradation.

Post-translational modifications, such as Ca^{2+} binding, phosphorylation, and lipidation, have roles in the regulation of annexin A1. Solito et al. [2006] showed that the translocation of annexin A1 from the cytoplasm to the cell surface is regulated by phosphorylation and lipidation. Annexin A1 is phosphorylated by several

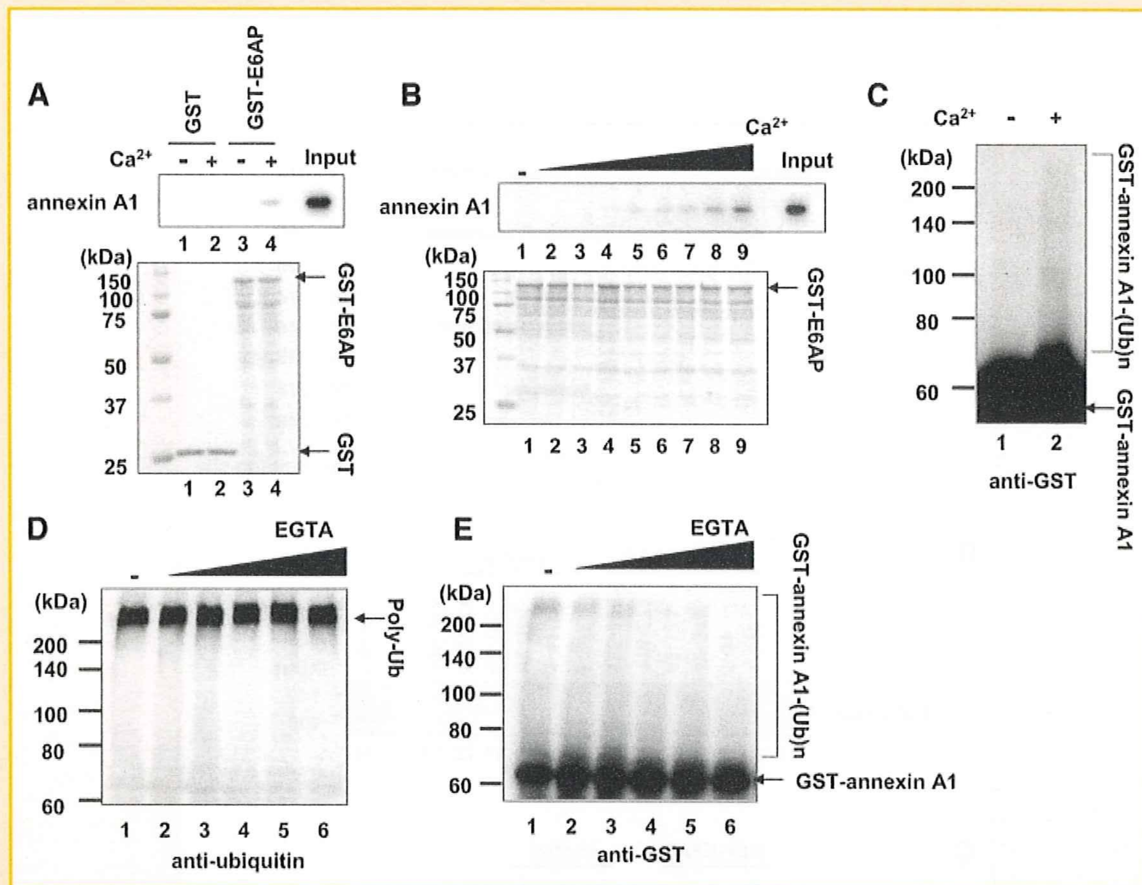

Fig. 6. E6AP mediates ubiquitylation of annexin A1 in a Ca^{2+}-dependent manner. A: In vitro binding of annexin A1 and E6AP. Immobilized GST-E6AP or GST alone was incubated with purified His-annexin A1 in the presence or absence of 1 mM $CaCl_2$ in the binding solution. Immunoblotting to detect bound annexin A1 was performed using anti-annexin A1 antibody. B: Ca^{2+}-dependent interaction between annexin A1 and E6AP. The GST pull-down assays described in (A) were repeated in the presence of increasing concentrations of $CaCl_2$ in the binding solution as follows: lane 1 (0 μM), 2 (10 μM), 3 (100 μM), 4 (250 μM), 5 (500 μM), 6 (750 μM), 7 (1 mM), 8 (2.5 mM), and 9 (5 mM). C: For in vitro ubiquitylation of annexin A1 protein, purified GST-annexin A1 was used as a substrate. Assays were done in 40-μl volumes in the presence or absence of 1 mM $CaCl_2$. The reaction mixture is described in Materials and Methods Section. The reaction was terminated by addition of SDS-PAGE loading buffer and followed by immunoblotting with anti-GST PAb. Arrow indicates GST-annexin A1. Ubiquitylated species of GST-annexin A1 proteins are marked by brackets. D,E: The in vitro ubiquitylation assays were performed in the presence of various concentrations of EGTA in the reaction mixture containing 1 mM $CaCl_2$. The concentrations of EGTA were as follows: lane 1 (0 mM), 2 (0.1 mM), 3 (0.5 mM), 4 (1 mM), 5 (5 mM), and 6 (10 mM). D: Immunoblotting to detect whole polyubiquitylated proteins with anti-ubiquitin MAb. E: Immunoblotting to detect polyubiquitylated GST-annexin A1 with anti-GST PAb.

protein kinases, such as epidermal growth factor receptor protein kinase, protein kinase C, and hepatocyte growth factor receptor kinase to mediate proliferation [Lim and Pervaiz, 2007], suggesting that phosphorylation plays some roles in the regulation of annexin A1 function. The findings presented in this study suggest that the ubiquitin-proteasome pathway plays a role in the regulation of annexin A1 function. Our data also suggest that E6AP preferentially recognizes the Ca^{2+}-binding form of annexin A1 and targets it for proteasomal degradation. The main biological property of annexin A1 is the binding to the phospholipid membrane in a Ca^{2+}-dependent manner [Lim and Pervaiz, 2007]. X-ray crystallography studies of annexin A1 have suggested that a calcium-driven conformational switch of the N-terminal and core domains of annexin A1 involves the membrane aggregation properties of annexin A1 [Rosengarth et al., 2001; Rosengarth and Luecke, 2003]. It will be intriguing to examine the role of E6AP in membrane aggregation. Further investigations will be required to elucidate the role of E6AP in the regulation of annexin A1 functions.

Targeting of a substrate via the ubiquitin system involves specific binding of the protein to the appropriate E3 ubiquitin ligase. There are several modes for specific substrate recognition, such as (1) NH2-terminal residue (N-end rule pathway), (2) allosteric activation, (3) recognition of phosphorylated substrate, (4) phosphorylation of E3, (5) phosphorylation of both the ligase and its substrate, (6) recognition in trans via an ancillary protein, (7) abnormal/mutated/misfolded proteins, and (8) recognition via hydroxylated proline [Glickman and Ciechanover, 2002]. E6AP specifically recognizes active forms of Blk, indicating that tyrosine phosphorylation of the regulatory tyrosine of Blk plays a role in specific substrate recognition [Oda et al., 1999]. Here we propose a novel mechanism of specific substrate recognition in the ubiquitin system, in which E6AP recognizes annexin A1 via a Ca^{2+}-induced conformational change. E6AP plays a direct catalytic role in the

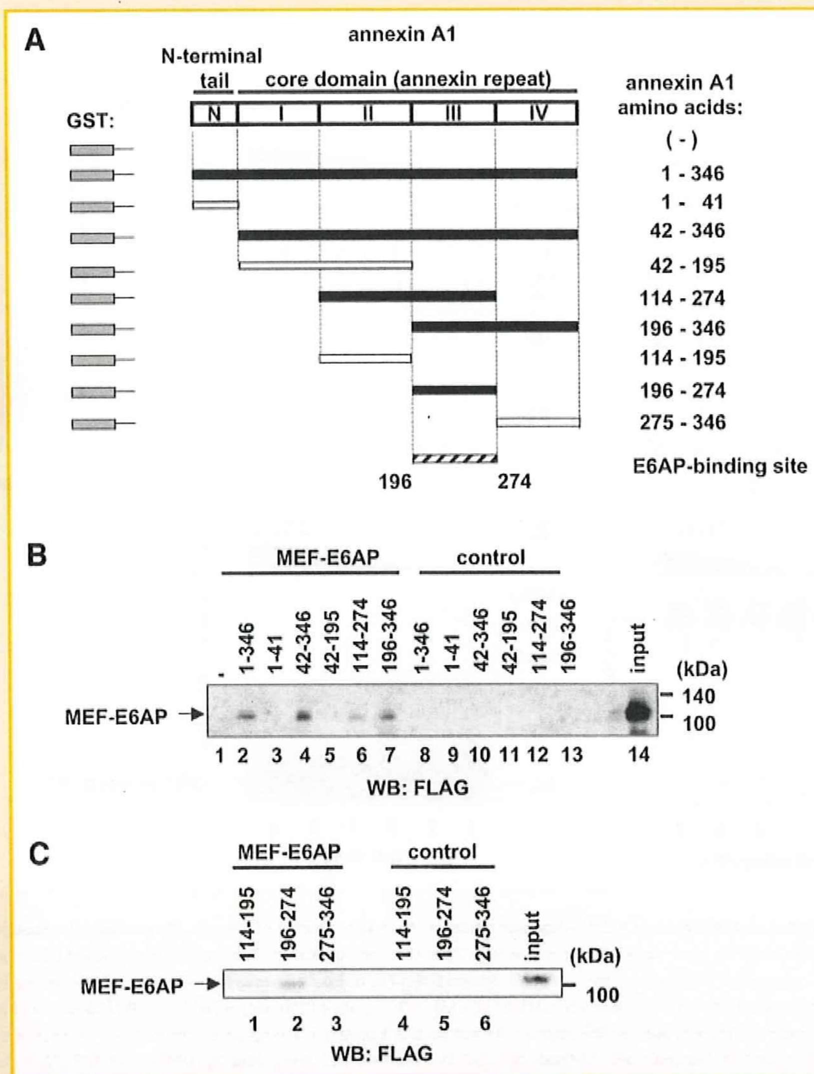

Fig. 7. Mapping of the E6AP-binding domain for annexin A1 protein. A: Structure of annexin A1. Shown is a schematic representation of the region of annexin A1 protein. N-terminal tail (aa 1–41), core domain (aa 42–346), and annexin repeat domains I (aa 42–113), II (aa 114–195), III (aa 196–274), and IV (aa 275–346) are shown. Schematic representation of GST-annexin A1 proteins. GST proteins contain the annexin A1 amino acids indicated to the right. The shaded box in each represents the GST sequence. Closed boxes represent proteins that are bound specifically to MEF-E6AP, and open boxes represent those that are not bound. B,C: In vitro binding of MEF-E6AP to GST-annexin A1 proteins. Purified recombinant MEF-E6AP was assayed for association with GST (−) or the GST-annexin A1 proteins using the binding buffer with 1 mM $CaCl_2$. GST pull-down was performed to assay for the association of E6AP with annexin A1. Control experiments were performed without MEF-E6AP. The association of MEF-E6AP was detected by immunoblotting with anti-FLAG MAb.

final attachment of ubiquitin to substrate proteins. Our findings suggest that Ca^{2+}-induced conformational change of annexin A1 may function as a degradation signal for annexin A1.

Ubiquitylated annexin A2 is enriched in the cytoskeleton fraction of mouse Krebs II cells [Lauvrak et al., 2005]. It remains unclear whether the ubiquitylated annexin A2 is degraded by proteasome. The apical membrane localization of Nedd4, a member of HECT-type ubiquitin ligases, is mediated by an association of its C2 domain with the apically targeted annexin XIIIb [Plant et al., 2000]. However, it is unknown whether annexin XIIIb is a substrate of Nedd4. To our knowledge, this is the first study to identify a specific E3 ubiquitin ligase for the ubiquitylation of an annexin family protein. All annexins share a core domain composed of four similar repeats, each approximately 70 amino acids long. Each repeat is composed of five α helices and usually contains a characteristic type-2 motif for binding calcium ions with the sequence GxGT-[38 residues]-D/E [Moss and Morgan, 2004]. The core domains of most vertebrate annexins reveal conservation of their secondary and tertiary structures despite the presence of only 45–55% amino-acid identity among individual annexins [Moss and Morgan, 2004]. It will be required to investigate whether other annexins are regulated by E6AP or other E3 ubiquitin ligases.

E6AP is hijacked by the HPV16E6 to target the tumor suppressor p53 in cervical cancer. Moreover, E6AP is mutated in Angelman syndrome and mediates ubiquitin-dependent degradation of HCV core protein, suggesting that E6AP plays important roles in sporadic and hereditary human diseases including cancer, neurological disorders, and infectious diseases [Kishino et al., 1997; Scheffner

and Staub, 2007; Shirakura et al., 2007]. Physical and functional association of E6AP with viral proteins, such as HPV16E6 [Huibregtse et al., 1993b] and HCV core protein [Shirakura et al., 2007], have been demonstrated. It is possible that the viral proteins redirect E6AP away from annexin A1, increasing the stability of annexin A1, and thereby contributing to viral pathogenesis. It would be interesting to investigate whether these viral proteins affect E6AP-dependent degradation of annexin A1. The association of E6AP with the viral protein (HPV16E6 or HCV core protein) could provide a feasible target for molecular approaches in the treatment of cervical cancer or HCV-related diseases.

In conclusion, we have demonstrated that E6AP interacts with annexin A1 protein and mediates its ubiquitin-dependent degradation. We propose that E6AP may play a role in regulating the diverse functions of annexin A1 protein. Identification of the specific E3 ubiquitin ligase may provide a link between the annexin family proteins and the ubiquitin-proteasome pathway. Elucidating the regulation of annexin A1 may provide a novel clue in the treatment of the E6AP-related diseases.

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Evaluation of Hepatitis C Virus Core Antigen Assays in Detecting Recombinant Viral Antigens of Various Genotypes▽

Mohsan Saeed,[1,3] Ryosuke Suzuki,[1] Madoka Kondo,[1] Hideki Aizaki,[1] Takanobu Kato,[1] Toshiaki Mizuochi,[2] Takaji Wakita,[1] Haruo Watanabe,[1,3] and Tetsuro Suzuki[1]*

Department of Virology II[1] and Department of Safety Research on Blood and Biological Products,[2] National Institute of Infectious Diseases, Tokyo 162-8640, and Department of Infection and Pathology, Graduate School of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033,[3] Japan

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A single substitution within the hepatitis C virus core antigen sequence, A48T, which is observed in ~30% of individuals infected with genotype 2a virus, reduces the sensitivity of a commonly used chemiluminescence enzyme immunoassay. Quantitation of the antigen is improved by using a distinct anticore antibody with a different epitope.

Hepatitis C virus (HCV) is a major cause of chronic liver disease throughout the world. Accurate diagnosis of HCV infection is important due to the morbidity associated with the virus, and determining the level of viral replication is important in predicting and monitoring the effect of antiviral treatment. Although quantifying viral RNA represents the standard method for identifying active infection (5, 8, 13), several sensitive immunoassays that detect the viral core antigen (Ag) have now been developed as an alternative to HCV RNA testing (3, 4, 6, 9, 10, 12, 16). The amino acid sequence of the core Ag is largely conserved among different viral isolates (14); however, genetic variability of the virus constitutes one of the major challenges to using core Ag assays for diagnosis. In this study, we examined the effects of sequence heterogeneity on the sensitivity of diagnostic kits for detection of the core Ag by using recombinant Ag derived from each of the major HCV genotypes. Expression plasmids for epitope-tagged core Ag were generated by inserting cDNA for the full-length core region of genotype 1a (17; GenBank accession no. AF011751), 1b (1; D89815), 2a (7; AB047639), 2b (AB030907), or 3a virus, with a FLAG tag sequence attached at its 5′ end, into the EcoRI site of the pCAG mammalian expression vector (11). HEK293T cells transiently transfected with the expression plasmids were harvested 48 h after transfection using a passive lysis buffer (Promega, Madison, WI). Centrifugation was performed to remove the debris after ultrasonication. Total protein was quantified in aliquots of cell lysate by using the bicinchoninic acid method (Pierce, Rockford, IL) and then used for determining the concentrations of HCV core Ag.

Figure 1A shows comparable levels of core Ag in each sample of cell lysate, as determined by immunoblotting with anti-FLAG antibody (Ab). The ability of HCV core Ag assays to detect five different HCV genotypes were compared using a commercially available chemiluminescence enzyme immunoassay (CLEIA) (Lumipulse II HCV core assay [assay detection range, approximately 50 to 50,000 fmol/liter]; Fujirebio, Japan) (15) and enzyme-linked immunosorbent assay (ELISA) (Ortho HCV Ag ELISA test [assay detection range, approximately 44.4 to 3,600 fmol/liter]; Ortho-Clinical Diagnostics, Japan) (2) to detect HCV core Ag in cell lysate. As shown in Fig. 1B,

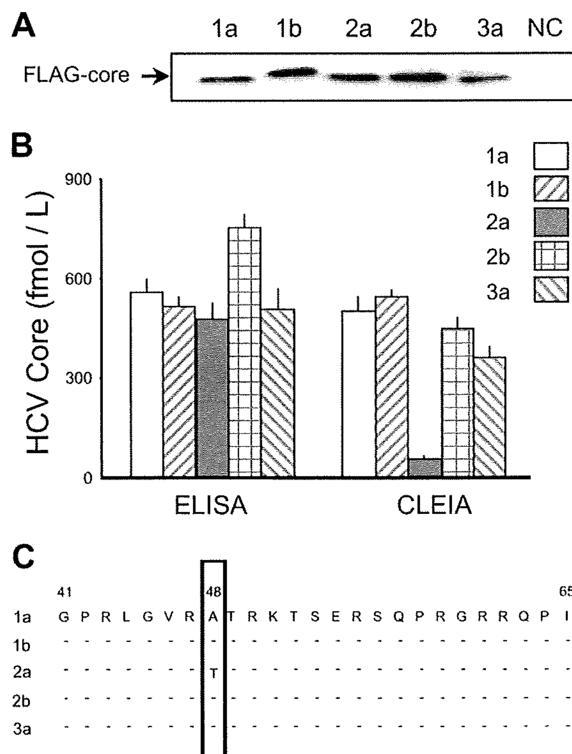

FIG. 1. Detection of recombinant HCV core Ag derived from genotype 1a, 1b, 2a, 2b, and 3a isolates by immunoblotting using an anti-FLAG Ab (A) as well as ELISA and CLEIA (B). The data shown in panel B represent the mean values and standard deviations ($n = 3$). NC, negative control. (C) The amino acid sequence from amino acids 41 to 65 of the core Ag used in this study. Key residues at the 48th position are boxed. Hyphens indicate conservation.

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1161. E-mail: tesuzuki@nih.go.jp.

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TABLE 1. Comparison of the 48th residues of HCV core Ags of genotypes 1a, 1b, 2a, 2b, and 3a

Genotype	No. of isolates	No. (%) of isolates with residue at 48th position		
		T	A	Other
1a	263	9 (3.5)	254 (96.5)	0 (0)
1b	298	2 (0.7)	294 (98.6)	2 (0.7)
2a	17	5 (29.5)	12 (70.5)	0 (0)
2b	17	0 (0)	17 (100)	0 (0)
3a	23	0 (0)	23 (100)	0 (0)
Total	618	16 (2.6)	600 (97.1)	2 (0.3)

although the ELISA measured similar concentrations of core Ag in all samples, apparent low levels of the genotype 2a core Ag, originally from an isolate known as the JFH-1 isolate (7), were detected using the CLEIA method, suggesting that some differences in the amino acid sequences corresponding to particular HCV genotypes or isolates may influence the sensitivity of core Ag detection. A comparison of the core Ag sequences, including the monoclonal Ab epitopes used in the development of CLEIA, revealed conservation of alanine at the 48th position in four clones, of genotypes 1a, 1b, 2b, and 3a, but not genotype 2a, for which there is a threonine at this position (Fig. 1C). Based on our analysis of sequences available from the HCV database (http://hcv.lanl.gov/content/sequence/NEWALIGN/align.html), alanine is highly conserved at the 48th residue of the core Ag for HCV isolates of genotypes 1a, 1b, 2b, and 3a (Table 1). In contrast, alanine and threonine occur in this position in 70.5% and 29.5%, respectively, of genotype 2a isolates. To examine whether the low sensitivity of the CLEIA method might be due to this particular amino acid change, we next replaced threonine with alanine at the 48th position of the JFH-1 core Ag (for

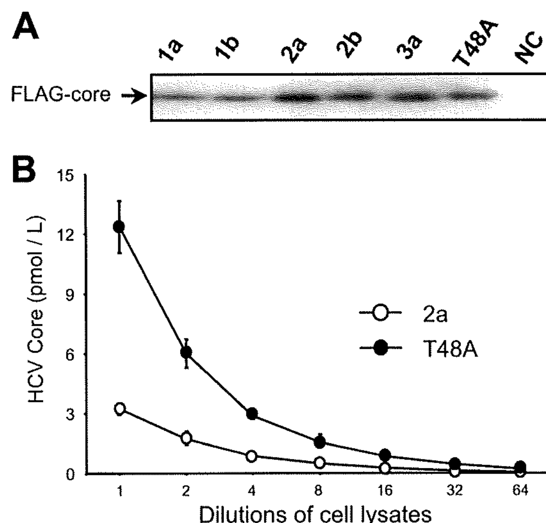

FIG. 2. Effect of T48A substitution in the core Ag of the JFH-1 isolate with regard to sensitivity of the CLEIA method. Samples of wild-type or mutated core Ag cell lysate were analyzed by immunoblotting (A) and CLEIA (B). The data shown in panel B represent the mean values and standard deviations (n = 3). NC, negative control.

TABLE 2. Comparison of the modified CLEIA with the original version for detection of the core Ags of genotypes 1a, 1b, 2a, 2b, and 3a[a]

Genotype	CLEIA	HCV core antigen concn (fmol/liter) in serially diluted cell lysates at indicated fold dilution							
		1	2	4	8	16	32	64	128
1a	Original	11,147	5,527	2,611	1,484	691	403	195	101
	Modified	10,511	5,700	2,676	1,420	716	444	200	111
1b	Original	11,612	5,618	3,081	1,551	779	409	223	113
	Modified	11,192	6,028	2,824	1,522	804	431	197	101
2a	Original	3,216	1,710	844	480	232	104	48	36
	Modified	12,101	6,255	3,153	1,676	805	422	212	106
2b	Original	10,559	5,635	2,811	1,286	762	387	194	94
	Modified	10,977	6,179	3,381	1,624	842	437	219	129
3a	Original	11,478	5,891	2,922	1,414	756	422	212	112
	Modified	11,208	6,225	3,126	1,555	791	445	215	100

[a] Data represent the mean values in triplicate measurements.

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the mutant JFH-1coreT48A) and measured the HCV core Ag concentration in cells expressing both mutated and wild-type JFH-1 core Ag. After confirming comparable levels of FLAG-tagged core Ag in the cell lysate samples by immunoblotting (Fig. 2A), HCV core Ag was quantified in the samples by serial dilution via the CLEIA method. As shown in Fig. 2B, the core Ag concentrations of JFH-1coreT48A were assessed to be 3.2- to 3.8-fold higher than those of the wild-type core Ag, suggesting that the sensitivity of HCV core Ag detection may have been affected by the 48th residue in the core Ag. Data for samples derived from genotypes 1a, 1b, 2b, and 3a were analogous to data for JFH-1coreT48A (data not shown). Although HCV isolates with threonine at the 48th position of the core Ag sequence comprise a relatively small proportion of the major genotype population, only 2.6% of the genotype 1a, 1b, 2a, 2b, and 3a isolates here (16 of 618 isolates; Table 1), attempts to overcome this problem would improve the overall sensitivity and usefulness of the assay. To achieve this aim, another monoclonal anticore Ab, whose epitope is comprised of amino acids 50 to 65, which are completely conserved among all the genotypes examined (Fig. 1C), was therefore used as a second Ab in a modified version of the CLEIA. We compared this modified assay with the original version by measurement of core Ag concentrations of the various genotypes (Fig. 2A) as illustrated in Table 2. The modified assay was able to quantify core Ag from genotypes 1a, 1b, 2a, 2b, and 3a with no significant differences observed between Ag levels in samples from different genotypes at each dilution.

It has been demonstrated that the HCV core Ag assay is a useful alternative to HCV RNA quantification for the diagnosis of hepatitis C and for monitoring the antiviral effects of treatment. Compared to various reverse transcription-PCR methods, HCV core assays are less expensive and easier to perform, without the requirement of sophisticated laboratory equipment and specially trained laboratory personnel. In addition, the core Ag assay can be used to measure a more diverse set of blood samples, such as sera stored for a long period of time, because the viral Ag is generally more stable than the RNA in sera or plasma. Despite the adequate performance of core Ag assays, we have shown that a single amino acid substitution at the 48th position of the core Ag changes the detection sensitivity. It is also noted that, although the original CLEIA should be improved, the ELISA used in this study may be substituted for it.

In conclusion, we have identified a distinct anticore Ab with a different epitope that might enable improved detection across all of the major HCV isolates. The findings of this study would provide useful information for the development of an improved assay with greater accuracy.

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消化器疾患最新の治療

[編集]
菅野健太郎 自治医科大学教授
上西紀夫 公立昭和病院院長
井廻道夫 昭和大学教授

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編集者　菅野健太郎，上西紀夫，井廻道夫
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4 B型慢性肝炎

鈴木文孝，熊田博光

I 疾患の解説

わが国におけるB型肝炎ウイルス(HBV)感染者はかつて人口の3%，300万人が存在するといわれていたが，近年母子感染事業による感染防御の実施や衛生環境の改善などによりHBVキャリアは減少傾向にある．実際，青年層でのキャリア率は低下し中高年でのキャリア率も1～2%と考えられている．わが国のHBVキャリアの多くは生後3年以内の免疫能の未熟な時期に感染したものであるが，その多くは出産時HBVキャリアの母親から感染することが多かった．現在は母親がHBVキャリアである場合は，γグロブリンとワクチン接種が行われており20歳以下のキャリア数は減少している．一方HBVのgenotype Aの感染例では成人でも慢性化する可能性があり，現在感染症例の増加が懸念されている．

HBVキャリアの自然経過は，若年時HBe抗原陽性でウイルス量が多いにもかかわらずALT値正常の時期(HBe抗原陽性の無症候性キャリア)から始まる．その後，宿主の免疫応答によってHBe抗体へとseroconversionを認め，最終的にはALT値正常，ウイルス量の低下したHBe抗体陽性の無症候性キャリアとなると考えられている．seroconversionの時期は10～30歳代に認められることが多く，女性のほうがより早い．seroconversionが起こる時期には多くの症例で一時的な肝炎の発症が認められる．しかし一部の症例では肝炎が持続し，慢性肝炎，肝硬変症へと進行する症例も認められる．またHBVキャリアの場合，無症候性キャリアとなっていても肝癌発癌の可能性はあり，定期的な経過観察は必要である．

II 診断と検査

HBVキャリアの病態を把握するうえで，HBV DNA量，HBe抗原の測定，肝機能検査は定期的に必要である．また初回検査時にはHBV genotypeを一度測定しておくことが望ましい(しかし現在保険適用が認められていない)．治療の目標は，HBe抗原の陰性化，ALT値の持続的正常化，HBV DNA量が5 log copies/mL以下を持続することである．最終的には，HBs抗原の陰性化が得られるとその後の肝炎の再燃はほとんど認められなくなり，発癌のリスクもかなり低下する．しかしわが国においてはHBVキャリアからのHBs抗原の陰性化はまれな現象である．

III 治療の一般方針

1 治療方針の立て方

B型慢性肝炎の治療に対しては，厚生労働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)における「B型及びC型肝炎ウイルスの感染者に対する治療の標準化に関する臨床的研究」の研究班においてB型慢性肝炎治療のガイドラインを作成している[1]．このガイドラインでは，年齢，HBe抗原の有無，ウイルス量によって分類し治療法を提示している．[illegible]若年層(35歳未満)は自己の免疫力によってHBe抗原の陰性化や肝炎の収束が期待されるため，核酸アナログ製剤の長期投与ではなくIFN長期間欠投与を基本治療としている．中高年では，核酸アナログ製剤の長期投与を基本治療としている．

2 薬物療法

a. インターフェロン(IFN)療法

わが国では，IFN療法はHBe抗原の慢性肝炎に対して6ヵ月間投与の保険治療が認められている．わが国でのIFN療法の治療成績は約20%が治療6ヵ月後の時点で著効となると報告され

表1　35歳未満B型慢性肝炎の治療ガイドライン

HBe抗原 ＼ HBV DNA量	≧7 log copies/mL	＜7 log copies/mL
e抗原陽性	①IFN長期投与(3ヵ月以上) ②エンテカビル	IFN長期投与(3ヵ月以上)
e抗原陰性	①経過観察 ②IFN長期投与(3ヵ月以上)あるいはエンテカビル	経過観察(F2以上の進行例にはIFN，エンテカビル)

治療対象は，ALT≧31 IU/Lで，HBe抗原陽性は，HBV DNA量5 log copies/mL以上，HBe抗原陰性は，4 log copies/mL以上

(文献1より引用)

表2　35歳以上B型慢性肝炎の治療ガイドライン

HBe抗原 ＼ HBV DNA量	≧7 log copies/mL	＜7 log copies/mL
e抗原陽性	①エンテカビル ②エンテカビル＋IFN連続投与(3ヵ月以上)	①エンテカビル ②IFN長期投与(3ヵ月以上)
e抗原陰性	エンテカビル	エンテカビル

治療対象は，ALT≧31 IU/Lで，HBe抗原陽性は，HBV DNA量5 log copies/mL以上，HBe抗原陰性は，4 log copies/mL以上

(文献1より引用)

ている[2]．著効になる症例は年齢が35歳未満，治療開始時ALT値が高い例であった．このことから，ガイドラインにおいても35歳未満のHBe抗原陽性症例では，IFN療法が推奨されている．

○ 処方例 ○ ••••••••••••••••••••

①スミフェロン　1日1回300万～600万単位，皮下または筋肉注射，2～4週間連日その後週3回合計24週間投与

②イントロンA　1週目1日1回600万～1,000万単位，2週目より1日1回600万，筋肉注射．ただし投与開始日は300万～600万単位を投与する
投与法は①と同じ

③オーアイエフ　1日1回250万～500万単位，筋肉注射
投与法は①と同じ

④フェロン　初日300万単位，点滴静注または静注，以後6日間1日1～2回，2週以降1日1回，点滴静注または静注

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b.　核酸アナログ製剤(ラミブジン，アデフォビル，エンテカビル)

核酸アナログ製剤であるラミブジンは，逆転写酵素阻害作用を有しウイルスのDNAポリメラーゼに選択的に作用する．ラミブジンは成人で腎機能が正常の症例には1日100 mg(ゼフィックス1錠)を経口投与する．ラミブジンには副作用がほとんど認められず，また強力なウイルス増殖抑制作用があり2000年の保険適用以来多くの症例で使用されてきた．しかしラミブジンは投与中止により多くの症例で肝炎の再燃を認めることと，長期投与によって耐性ウイルスが高率に出現するという問題点がある．このため現在ではより耐性ウイルスの出現率が低いエンテカビル(バラクルード)の使用が奨励されている．

○ 処方例 ○ ••••••••••••••••••••

①ゼフィックス(100 mg)　1錠/日(分1)，経口投与．原則的に長期投与を行う

ラミブジン耐性ウイルス出現例の対処としては，アデフォビルまたはエンテカビルの使用が可能である．この場合アデフォビルの使用が奨励さ

表3 ラミブジン投与中B型慢性肝炎患者に対する核酸アナログ製剤治療ガイドライン

HBV DNA量 ＼ ラミブジン投与期間		3年未満	3年以上
<2.6 log copies/mL 持続		エンテカビル0.5 mg/日に切り替え可	ラミブジン100 mg/日を継続
≧2.6 log copies/mL	BTH* なし	エンテカビル**0.5 mg/日に切り替え可	ラミブジン100 mg/日を継続
≧2.6 log copies/mL	BTH* あり	アデフォビル10 mg/日併用	アデフォビル10 mg/日併用

* BTH : breakthrough hepatitis

** ラミブジン変異のないことを確認後投与

（文献1より引用）

れている．アデフォビルはエンテカビルと比較して，HBV DNAの減少率で上回り，またラミブジンとの併用で両剤への耐性ウイルスの出現率が低率であることが報告されている．一方エンテカビルの場合は，ラミブジン耐性ウイルスが存在する症例ではエンテカビル耐性ウイルスの出現の可能性があるためその使用には慎重でなくてならない．

○ 処方例 ○

①ヘプセラ（10 mg） 1錠/日（分1），経口投与．ラミブジン耐性ウイルス出現時にラミブジン100 mgと併用投与する

エンテカビルは2006年9月に保険適用となった新たな核酸アナログ製剤である．エンテカビルは，ラミブジンと比較してHBV DNAの減少率で有意に上回っていること，また核酸アナログ未使用症例（naive症例）においては耐性ウイルスの出現率が低率であることが報告されている．このことからガイドラインではnaive症例における第一選択薬となっている．一方前述したようにラミブジン耐性ウイルスが出現した症例では，エンテカビル投与によってエンテカビル耐性ウイルスの出現の可能性があり，エンテカビルの使用は慎重でなくてはならない．

○ 処方例 ○

①バラクルード（0.5 mg） 1錠/日（分1）（内服前後で2時間は食事摂取を避ける必要がある．このため眠前に投与することが多い），経口投与

②バラクルード（1.0 mg） 1錠/日（分1），経口投与．ラミブジン耐性ウイルス出現例では1.0 mg投与が可能である．この場合ラミブジンから切り替える必要がある．

エンテカビルの登場によって核酸アナログ製剤の使用法も変化してきている．もともとラミブジンを使用していた症例での核酸アナログ製剤使用の方法についてもガイドラインは提示している（表3）．HBV DNA量が2.6 log copies/mL以上でbreakthrough hepatitis（BTH）を認めた症例はラミブジンとアデフォビル併用．BTHを認めていない3年未満の症例ではできればラミブジン耐性ウイルスの有無を確認し耐性ウイルスが認められなければエンテカビルに切り替え可能，3年以上経過している症例では耐性ウイルスが認められる可能性が高いことからラミブジンを継続しBTH出現時にはアデフォビルを併用することとしている．HBV DNA量2.6 log copies/mL未満の症例のうち，投与期間が3年未満ではラミブジン耐性ウイルスの存在する可能性が低いためエンテカビルに切り替え可能であるが，3年以上投与例では耐性ウイルスが存在している可能性が高くなるためラミブジン継続としている．

3 その他

ガイドラインの補足として，治療の対象や治療に対する基本的な考え方が提示されている（表4）．B型慢性肝炎の経過は個々の症例で違いがあり，年齢，ウイルスマーカー，肝組織所見，家族歴などを考慮し総合的に決定する必要がある．

表 4 B 型慢性肝炎の治療(ガイドラインの補足)

1. 抗ウイルス療法は，ALT 値が ALT≧31 IU/L の場合に考慮する．しかし高齢者や HBe 抗原陰性例，抗ウイルス薬の投与が難しい例では肝庇護療法(SNMC，UDCA など)で経過をみることも可能である．
2. 若年(35 歳未満)症例では，インターフェロン(IFN)長期投与(3 ヵ月以上)あるいはエンテカビル投与が原則．なお，ステロイド，IFN，核酸アナログの短期併用療法も考慮すること．ただし組織像の軽い症例では自然経過一の HBe 抗原の seroconversion を期待し follow-up することもある．(IFN 在宅自己注射可能な症例は QOL を考慮し在宅自己注射を推奨する)
3. 中高年(35 歳以上)症例では，核酸アナログ未使用例は，エンテカビルが第一選択になる．
4. 母子感染例は IFN 抵抗性のことが多く，エンテカビル単独あるいはエンテカビル＋IFN 連続療法も適応になる．
5. ラミブジン耐性ウイルスによる肝炎に対しては，アデフォビルが第一選択になる．組織学的進行例では HBV DNA 量が上昇した時点でアデフォビルを開始する．
6. 肝硬変例および肝細胞癌治癒後の症例も，核酸アナログの治療を行う．

注意

1. HIV を合併している症例では，エンテカビルの使用により HIV 耐性ウィルスが出現する可能性があるためエンテカビルは使用できない．
2. HBV DNA 量が低値で ALT 値が正常の例であっても免疫抑制作用のある薬剤や抗がん剤投与時には HBV DNA 量が上昇して高度の肝障害をきたすことがあるため注意が必要である(免疫抑制・化学療法により発症する B 型肝炎に対する診療ガイドライン参照)．

(文献 1 より引用)

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1) 熊田博光：厚生労働科学研究費補助金，肝炎等克服緊急対策研究事業(肝炎分野)，肝硬変を含めたウイルス性肝疾患の治療の標準化に関する研究，平成 19 年度総括・分担研究報告書，平成 20 年 3 月
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A Matched Case-Controlled Study of 48 and 72 Weeks of Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype 1b in Japan: Amino Acid Substitutions in HCV Core Region as Predictor of Sustained Virological Response

Norio Akuta,[1]* Fumitaka Suzuki,[1] Miharu Hirakawa,[1] Yusuke Kawamura,[1] Hiromi Yatsuji,[1] Hitomi Sezaki,[1] Yoshiyuki Suzuki,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Mariko Kobayashi,[2] Satoshi Saitoh,[1] Yasuji Arase,[1] Kenji Ikeda,[1] and Hiromitsu Kumada[1]

[1]*Department of Hepatology, Toranomon Hospital, Tokyo, Japan*
[2]*Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan*

Substitution of amino acid (aa) 70 and 91 in the core region of HCV genotype 1b is a useful pretreatment predictor of efficacy of 48-week peginterferon (PEG-IFN) plus ribavirin (RBV) therapy. Here, we determined the efficacy of 72-week PEG-IFN/RBV and the predictive factors to such therapy in a case–control study matched for sex, age, and periods from the start of treatment to initial point of HCV RNA-negative. We compared the treatment efficacy of 72-week regimen in 65 patients with that of 48-week in 130 patients, who were infected with HCV genotype 1b and treated with PEG-IFN/RBV. They consisted mainly of late virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after start of treatment). Sustained virological response (SVR) was achieved by 61.5% and 32.3% of patients of the 72- and 48-week groups, respectively, while non-virological response was noted in 9.2% and 29.2% of the respective groups. Multivariate analysis identified substitution of aa 70 and 91 (Arg70 and/or Leu91) and duration of treatment (72-week) as independent parameters that significantly influenced SVR. For Arg70 and/or Leu91 of core region, SVR rate was significantly higher in 72- (68.0%) than 48-week group (37.8%). For wild-type of ISDR, SVR rate was significantly higher in 72- (61.2%) than in 48-week group (29.3%). We conclude that 72-week PEG-IFN/RBV improves SVR rate for LVR, especially those with Arg70 and/or Leu91 of core region or wild-type of ISDR. Substitution of aa 70 and 91 is also a useful pretreatment predictor of response to 72-week PEG-IFN/RBV. ***J. Med. Virol. 81:452–458, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; core region; NS5A-ISDR; peginterferon; ribavirin; 72-week; case–control study; LVR

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INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. In patients with HCV-chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989]. Especially, peginterferon (PEG-IFN) plus ribavirin (RBV) combination therapy for 48 weeks can achieve a high sustained virological response (SVR) [Manns et al., 2001; Fried et al., 2002].

Although treatment of genotype 1-infected patients typically extends over 48 weeks, there has been interest in prolongation of therapy, particularly in late

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*Correspondence to: Norio Akuta, MD, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp

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virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment), because high relapse rates in LVR may indicate that treatment was not administered for a sufficient duration [Ferenci et al., 2005]. Previous studies from Europe and United States have demonstrated that LVR improves SVR rates when treatment is extended to 72 weeks, compared with standard duration of therapy, largely as a result of reducing posttreatment relapse rates [Buti et al., 2003; Berg et al., 2006; Sánchez-Tapias et al., 2006; Pearlman et al., 2007]. Thus, prolongation of therapy in LVR may improve the virological response rate. However, it is not clear at present whether prolongation of treatment improves the SVR rate of treatment-resistant Japanese patients infected with HCV/genotype 1b [Akuta et al., 2007a,b,c].

Previous studies indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b were predictors of poor virological response to 48-week PEG-IFN plus RBV therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also risk factors for hepatocarcinogenesis [Akuta et al., 2007d, 2008a]. However, it is not clear at this stage whether aa substitutions in the core region can be used before therapy to predict the outcome of 72-week regimen.

The aims of the present study in HCV genotype 1b-infected Japanese adult patients, who received PEG-IFN plus RBV, were the following: (1) To conduct a case–control study matched for sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative, to compare the treatment efficacy of 72-week regimen and 48-week regimen. (2) To identify the pretreatment factors that could predict treatment efficacy of the 72-week regimen, including pretreatment aa substitutions in the core region.

PATIENTS AND METHODS

Study Population

A total of 559 HCV genotype1b-infected Japanese adult patients were consecutively recruited into the study protocol of combination therapy with PEG-IFNα-2b plus RBV between 2001 and 2008 at Toranomon Hospital, Tokyo, Japan. They received PEG-IFNα-2b at a median dose of 1.4 μg/kg (range, 0.7–2.1 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.1 mg/kg (range, 3.4–16.0 mg/kg) daily. Among these, 383 patients, who could complete a total of 48 or 72 weeks of combination therapy, were enrolled in this retrospective study. The latter group consisted of 65 patients who extended combination therapy to 72-week (72-week group), and 318 patients who stopped combination therapy at the 48 weeks (48-week group). The decision to extend the combination therapy to 72 weeks was made by the patient. To compare the efficacy of the 72- and 48-week courses, all 65 patients of the 72-week group entered this study along with 130 patients of 48-week. The latter group was selected from among the 318 because they matched those patients of the 72-week group with respect to sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative (matched case–control study). The treatment efficacy was evaluated by HCV-RNA positive based on qualitative PCR analysis at the end of treatment (non-virological response; NVR), and by HCV-RNA negative based on qualitative PCR analysis at 24 weeks after the completion of therapy (SVR). Furthermore, LVR was defined as HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment, based on qualitative PCR analysis. All patients fulfilled the following criteria: (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostics, Manheim, Germany). (2) Infection with HCV genotype 1b only. (3) A high viral load ($\geq 100 \times 10^3$ IU/ml) by quantitative analysis of HCV RNA with PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc., Pleasanton, CA) within the preceding 2 months of enrolment. (4) No hepatocellular carcinoma. (5) Body weight > 40 kg. (6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg. (9) None had other forms of liver diseases, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, or autoimmune liver disease. (10) None of the females was pregnant or a lactating mother. (11) All patients had completed a 24-week follow-up program after cessation of treatment, and SVR could be evaluated. (12) Each signed a consent form of the study protocol that had been approved by the human ethics review committee. The profile and laboratory data of 195 patients, who entered the matched case–control study, are summarized in Table I.

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured by quantitative PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc.) at least once every month before, during, and after therapy. The dynamic range of the assay was 5.0×10^3 to 5.0×10^6 IU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA ($<5.0 \times 10^3$ IU/ml) were also checked by qualitative PCR (AMPLICOR HCV v2.0, Roche Molecular Systems Inc.),

TABLE I. Patient Profile and Laboratory Data at Commencement of 48- and 72-Week Combination Therapy of Peginterferon Plus Ribavirin in Patients Infected With HCV Genotype 1b (Matched Case–Control Study)

	72-week group	48-week group	
Matching data			
Number of patients	65	130	
Sex (M/F)	28/37	57/73	Matched
Age (years)*	57 (22–70)	56 (25–68)	Matched
Periods to the initial point of HCV RNA-negative (weeks)*	17.4 (5.9–72.0)	19.7 (6.0–48.0)	Matched
Demographic data			
History of blood transfusion	18 (27.7%)	42 (32.3%)	NS
Family history of liver disease	21 (32.3%)	31 (23.8%)	NS
Body mass index (kg/m^2)*	22.6 (16.6–38.0)	22.2 (17.0–32.4)	NS
Laboratory data*			
Serum aspartate aminotransferase (IU/L)	49 (23–213)	51 (21–217)	NS
Serum alanine aminotransferase (IU/L)	64 (25–430)	68 (20–391)	NS
Serum albumin (g/dl)	3.9 (3.2–4.5)	3.8 (3.2–4.6)	NS
Gamma-glutamyl transpeptidase (IU/L)	40 (14–171)	38 (15–581)	NS
Leukocytes (/mm^3)	4,400 (2,300–8,800)	4,600 (1,200–9,400)	NS
Hemoglobin (g/dl)	14.0(11.3–17.8)	13.9 (10.6–18.1)	NS
Platelet count ($\times 10^4$/mm^3)	16.2 (8.2–30.7)	15.8 (6.4–31.6)	NS
ICG R15 (%)	13 (2–73)	15 (2–45)	NS
Level of viremia (KIU/ml)	2,650 (52->5,000)	1,850 (49->5,000)	0.013
Alfa-fetoprotein (μg/L)	6 (2–47)	6(2–110)	NS
Total cholesterol (mg/dl)	174(111–276)	175 (104–274)	NS
High density lipoprotein cholesterol (mg/dl)	45 (27–86)	51 (24–78)	NS
Low density lipoprotein cholesterol (mg/dl)	104 (49–204)	107 (50–182)	NS
Triglycerides (mg/dl)	91 (35–259)	94 (35–315)	NS
Uric acid (mg/dl)	5.3 (2.6–7.7)	5.0 (2.3–8.7)	NS
Fasting blood sugar (mg/dl)	95 (79–218)	98 (76–157)	NS
Histological findings			
Stage of fibrosis (F1/F2/F3/ND)	20/12/11/1/21	44/27/22/0/37	NS
Hepatocyte steatosis (none to mild/moderate to severe/ND)	40/2/23	78/8/44	NS
Treatment			
PEG-IFN α-2b dose (μg/kg)*	1.4 (0.8–2.1)	1.4 (0.7–1.9)	NS
Ribavirin dose (mg/kg)*	10.9 (6.6–16.0)	10.8 (3.7–14.2)	NS
Amino acid substitutions in the HCV			
Core aa 70 (arginine/glutamine (histidine)/ND)	37/23/5	11 47/6	NS
Core aa 91 (leucine/methionine/ND)	42/18/5	66/57/7	0.038
ISDR of NS5A (wild-type/mutant-type/ND)	49/5/11	99/17/14	NS

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values. ND: not determined.

which has a higher sensitivity than quantitative analysis, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examinations contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was graded as either none (absent), mild (less than 1/3 of hepatocytes involved), moderate (greater than 1/3 but less than 2/3 of hepatocytes involved), or severe (greater than 2/3 of hepatocytes involved) [D'Alessandro et al., 1991].

Detection of Amino Acid Substitutions in Core Region and NS5A Region

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of genotype 1b was determined and then compared with the consensus sequence constructed on 50 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005]. The sequence of 2209–2248 aa in the NS5A of genotype 1b (IFN-sensitivity determining region [ISDR]) reported by Enomoto et al. [1995, 1996] was also determined, and the numbers of aa substitutions in ISDR were defined as wild-type (≤ 1) or mutant-type (≥ 2).

In the present study, aa substitutions of the core region and NS5A-ISDR were analyzed by direct sequencing [Enomoto et al., 1995, 1996; Akuta et al., 2005]. HCV RNA was extracted from serum samples at

the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by PCR using the following primers: (a) Nucleotide sequences of the core region: The first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of NS5A-ISDR: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers ([a] hemi-nested PCR; [b] nested PCR). All samples were initially denatured at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

Statistical Analysis

Non-parametric tests (Mann–Whitney *U*-test, chi-squared test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to SVR. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All *P* values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. The potential pretreatment predictive factors associated with SVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, aspartate aminotransferase (AST), ALT, albumin, gamma-glutamyl transpeptidase (γGTP), leukocyte count, hemoglobin, platelets, indocyanine green retention rate at 15 min (ICG R15), level of viremia, alfa-fetoprotein, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, uric acid, fasting blood sugar, hepatocyte steatosis, stage of fibrosis, PEG-IFN dose/body weight, RBV dose/body weight, duration of treatment, and amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Comparison of Treatment Efficacy Between 48-Week Group and 72-Week Group

Figure 1 shows comparison of the treatment efficacy between 48- and 72-week groups. SVR was achieved by 42 of 130 patients (32.3%) and 40 of 65 (61.5%) in the 48- and 72-week groups, respectively. The proportion of SVR was significantly higher in 72-week group than in the 48-week group ($P < 0.001$). Furthermore, NVR was identified in 38 of 130 patients (29.2%) and 6 of 65 (9.2%) in the 48- and 72-week groups, respectively. The proportion of NVR was significantly lower in the 72-week group than in 48-week group ($P = 0.002$).

Predictive Factors Associated With SVR in Multivariate Analysis

Univariate analysis identified 13 parameters that influenced SVR either significantly or marginally: gender (female sex; $P = 0.002$), stage of fibrosis (F_{1-2}; $P = 0.008$), PEG-IFN dose/body weight (≥1.4 μg/kg; $P = 0.001$), RBV dose/body weight (≥11.0 mg/kg; $P = 0.029$), platelet count ($\geq 15.0 \times 10^4$/mm^3; $P = 0.002$), level of viremia (<1,000 KIU/ml; $P = 0.049$), γGTP (<50 IU/L; $P = 0.026$), ICG R15 (<15%; $P = 0.003$), triglycerides (<100 mg/dl; $P = 0.038$), high-density lipoprotein cholesterol (≥50 mg/dl; $P = 0.018$), α-fetoprotein (<20 μg/L; $P = 0.005$), substitution of aa 70 and 91 (Arg70 and/or Leu91; $P = 0.002$), and duration of treatment (72-week group; $P < 0.001$).

Multivariate analysis identified three independent parameters that either significantly influenced or tended to significantly influence SVR; substitution of aa 70 and 91 (Arg70 and/or Leu91; $P = 0.015$), duration of treatment (72-week group; $P = 0.014$), and high-density lipoprotein cholesterol (≥50 mg/dl; $P = 0.084$) (Table II).

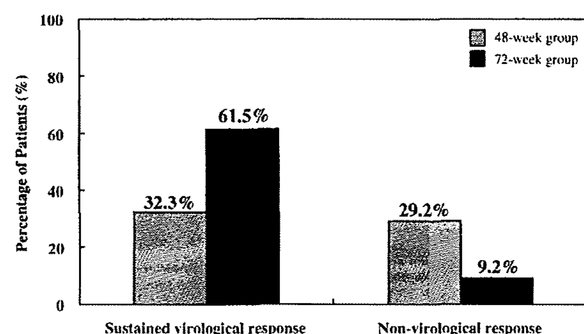

Fig. 1. Comparison of treatment efficacy between the 48-week group and 72-week group. The proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P < 0.001$). Furthermore, the proportion of patients with non-virological response in 72-week group was significantly lower than in 48-week group ($P = 0.002$).

TABLE II. Factors Associated With Sustained Virological Response to Combination Therapy of Peginterferon Plus Ribavirin in 195 Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
Substitution of aa 70 and 91	1: Gln70 (His70) and Met91	1	
	2: Arg70 and/or Leu91	5.46 (1.39–21.3)	0.015
Duration of treatment (weeks)	1: 48	1	
	2: 72	3.51 (1.28–9.62)	0.014
HDL-cholesterol (mg/dl)	1: <50	1	
	2: ≥50	2.42 (0.89–6.58)	0.084

*Only variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on multivariate logistic regression are shown.

Treatment Efficacy According to Amino Acid Substitutions in Core Region

Figure 2 shows comparison of the treatment efficacy according to aa substitutions in the core region. In Gln70 (His70) and Met91, SVR was achieved by 4 of 26 patients (15.4%) and 3 of 10 (30.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was not significantly different than in 48-week group. In Arg70 and/or Leu91, SVR was achieved by 37 of 98 patients (37.8%) and 34 of 50 (68.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was significantly higher than in 48-week group ($P = 0.001$).

Treatment Efficacy According to Amino Acid Substitutions in NS5A-ISDR

Figure 3 shows comparison of the treatment efficacy according to aa substitutions in NS5A-ISDR. In mutant-type, SVR was achieved by 9 of 17 patients (52.9%) and 3 of 5 (60.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was not significantly different from that in 48-week group. In wild-type, SVR was achieved by 29 of 99 patients (29.3%) and 30 of 49 (61.2%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was significantly higher than that in 48-week group ($P < 0.001$).

DISCUSSION

This matched case-controlled study of PEG-IFN plus RBV for LVR infected with HCV genotype 1b, showed that treatment extension to 72 weeks seems to improve SVR rates in Japanese patients. To our knowledge, the present study is the first to report that 72-week regimen of PEG-IFN plus RBV might be also useful in Asians. Especially, the 72-week regimen significantly improved the SVR rates in LVR with Arg70 and/or Leu91 of core or wild-type of ISDR. The present study based on patients, who could complete a total of 48 or 72 weeks of combination therapy, did not show the frequencies of patients, who could not complete by side effects. Patients, who dropped out by side effects between 48 and 72 week for therapy prolonged to 72 weeks, were only 3 of 559 HCV genotype1b-infected Japanese adult patients (data not shown), so the frequencies of side effects with 72-week regimen might be nearly equal to those with 48-week regimen. Large-scale prospective study based on the intention to treat analysis should be conducted to confirm the above finding in future.

NS5A-ISDR, reported as predictor of treatment efficacy with IFN monotherapy by Enomoto et al. [1995, 1996], is also useful as predictor of 48-week PEG-IFN plus RBV combination therapy [Murayama et al., 2007; Shirakawa et al., in press; Yen et al., 2008]. Furthermore, the present study also indicated that

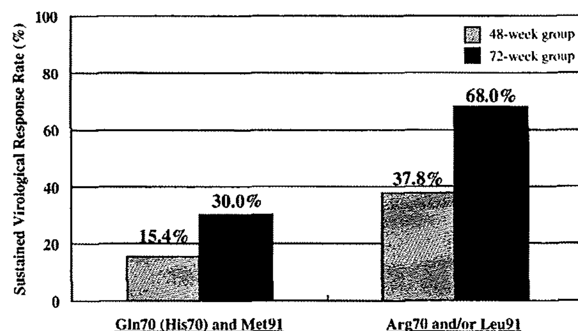

Fig. 2. Comparison of treatment efficacy according to amino acid substitutions in the core region. In Gln70 (His70) and Met91, the proportion of patients with sustained virological response in 72-week group was not significantly different from that in 48-week group. However, in Arg70 and/or Leu91, the proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P = 0.001$).

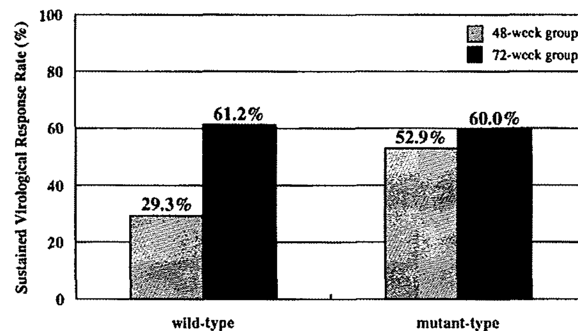

Fig. 3. Comparison of treatment efficacy according to amino acid substitutions in NS5A-ISDR. In mutant-type, the proportion of patients with sustained virological response in 72-week group was not significantly different from that in 48-week group. However, in wild-type, the proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P < 0.001$).

72-week regimen of PEG-IFN plus RBV significantly improved the SVR rate in LVR with wild-type of ISDR. Unfortunately, the 72-week regimen of PEG-IFN plus RBV did not improve the SVR rate in LVR with Gln70 (His70) and Met91 of the core region. Multivariate analysis also identified Gln70 (His70) and Met91 of the core region as independent parameter that significantly influenced non-SVR. PEG-IFN plus RBV carries potential serious side effects and is costly especially when used long enough to achieve higher SVR rates. For these reasons, we need to identify those patients who do not achieve SVR, to free them of unnecessary side effects and reduce costs, preferably before the start of the combination therapy. For patients unsuitable for PEG-IFN plus RBV, including LVR with Gln70 (His70) and Met91 of the core region, low-dose intermittent IFN monotherapy might be an efficacious therapeutic regimen, because it can lead to ALT normalization and thus reduce the risk of hepatocarcinogenesis [Akuta et al., 2008b].

One limitation of this study is that LVR could not be evaluated by the COBAS AmpliPrep/COBAS TaqMan HCV Test (the lower limit of this assay; 15 IU/ml), which has a higher sensitivity than AMPLICOR HCV v2.0 (the lower limit of this assay; 50 IU/ml) [Sizmann et al., 2007]. Rapid virological response (HCV RNA-negative at 4 weeks after the start of treatment) and early virological response (HCV RNA-positive at 4 weeks and negative at 12 weeks after the start of treatment) by AMPLICOR HCV v2.0 might be diagnosed as LVR by the COBAS AmpliPrep/COBAS TaqMan HCV Test. Further studies using highly sensitive real-time PCR assay should be performed to facilitate the development of more effective therapeutic regimens in future.

We previously reported that viral factors (e.g., aa substitutions in core region) and host factors (e.g., lipid metabolic factors, sex, and AFP) might be important predictors of treatment response to 48-week PEG-IFN plus RBV in Japanese patients infected with HCV genotype 1b, in addition to treatment-related factors (e.g., RBV dose) [Akuta et al., 2005, 2006, 2007a,b,c]. The present study also identified viral (aa substitutions in the core region), host (HDL-cholesterol), and treatment-related factors (duration of treatment) that can be useful as independent and significant pretreatment predictors of SVR. Thus, substitution of aa 70 and 91 is also useful as a pretreatment predictor of 72-week regimen. Further studies that examine the structural and functional impact of aa substitutions during combination therapy should be conducted to confirm the above finding.

Another limitation of our study was that we did not examine aa substitutions in areas other than the core region and NS5A-ISDR of HCV genome, such as the interferon/ribavirin resistance determining region (IRRDR), including V3 of NS5A region, although they should be investigated in future studies [El-Shamy et al., 2008; Muñoz de Rueda et al., 2008].

We conclude that treatment efficacy of 72-week PEG-IFN plus RBV seems to be based on a dynamic tripartite interaction of viral-, host-, and treatment-related factors. Further understanding of the complex interaction between these factors should facilitate the development of more effective therapeutic regimens.

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