Table 2
List of 11 transcripts whose expression levels are positively correlated with age (AR-increase).

Agilent Probe ID	Gene name	Gene symbol	Gene ID	Site H		Site M		Correlation average	Expected cell types
				Correlation coefficient	FDR	Correlation coefficient	FDR		
A_24_P18137	Neurofilament, light polypeptide	NEFL	4747	0.505	2.17E-03	0.480	1.06E-01	0.492	Memory T-resting (naïve)[a]
A_23_P44674	Cysteine-rich protein 1 (intestinal)	CRIP1	1396	0.419	3.36E-02	0.468	1.07E-01	0.443	NA
A_32_P186731	Isthmin 1 homolog (zebrafish)	ISM1	140862	0.492	2.35E-03	0.391	2.13E-01	0.442	Memory T-resting (naïve)[a], CD4 T cell (naïve and resting)[a]
A_23_P62959	Pleckstrin homology-like domain, family A, member 3	PHLDA3	23612	0.498	2.17E-03	0.385	2.13E-01	0.441	Macrophage-7 day (of differentiatior
A_23_P215048	KIAA0408	KIAA0408	9729	0.419	3.36E-02	0.424	1.99E-01	0.422	B cell (naïve), Helper Th1-12 h (of differentiation)
A_23_P52610	Damage-specific DNA binding protein 2, 48 kDa	DDB2	1643	0.447	1.56E-02	0.396	2.13E-01	0.421	Helper Th2-48 h (of differentiation)
A_23_P92073	Poly(ADP-ribose) polymerase family, member 3	PARP3	10039	0.426	3.08E-02	0.405	2.07E-01	0.415	NA
A_23_P79482	Chimerin (chimaerin) 1	CHN1	1123	0.403	6.26E-02	0.369	2.44E-01	0.386	Memory T-resting (naïve)[a], Memory T-activated[a]
A_23_P391228	Mannosidase, endo-alpha-like	MANEAL	149175	0.381	1.10E-01	0.385	2.13E-01	0.383	NA
A_23_P23924	Calpain 2, (m/II) large subunit	CAPN2	824	0.347	2.12E-01	0.416	2.03E-01	0.382	NA
A_24_P383649	Archaelysin family metallopeptidase 1	AMZ1	155185	0.373	1.30E-01	0.364	2.49E-01	0.368	NA

Correlation coefficients were independently obtained with the site H dataset and site M datasets. Transcripts are arranged according to the "Correlation Average" from the two sites. Assignment of source cells expressing these transcripts are noted in the Materials and methods section.
[a] Over 3 IQR of the upper quartile.

Table 3
List of five transcripts whose expression levels are negatively correlated with age (AR-decrease).

Agilent Probe ID	Gene name	Gene symbol	Gene ID	Site H		Site M		Correlation average	Expected cell types
				Correlation coefficient	FDR	Correlation coefficient	FDR		
A_23_P52697	CD248 molecule, endosialin	CD248	57124	−0.604	3.80E-06	−0.467	1.07E-01	−0.535	CD8 T cell (naïve and resting)[a]
A_24_P314786	Solute carrier family 4, sodium bicarbonate transporter, member 10	SLC4A10	57282	−0.494	2.35E-03	−0.518	4.28E-02	−0.506	B cell (naïve), Memory B-IgM
A_24_P930111	Solute carrier family 4, sodium bicarbonate transporter, member 10	SLC4A10	57282	−0.485	2.90E-03	−0.496	8.62E-02	−0.491	B cell (naïve), Memory B-IgM
A_24_P348806	Pleckstrin homology domain containing, family A member 7	PLEKHA7	144100	−0.350	2.08E-01	−0.479	1.06E-01	−0.415	Plasma B-PBMC [a], Plasma B-bone marrow
A_23_P32444	Matrix-remodelling associated 8	MXRA8	54587	−0.369	1.38E-01	−0.364	2.49E-01	−0.366	Plasma B-bone marrow

Correlation coefficients were independently obtained with the site H dataset and site M dataset. Transcripts are arranged according to the "Correlation Average" from the two sites. Assignment of source cells expressing these transcripts are noted in the Section 2.
[a] Over 3 IQR of the upper quartile.

3. Results

3.1. Clinical characteristic of the two cohorts

Demography of participants in two cohorts of healthy individuals is shown in Table 1. Site H consisted of 46 males and 44 females. Site M consisted of 35 males and 29 females. The balance of gender is deemed reasonable. At site H, the average age of subjects was 40 ± 10, the age range from 23 to 66. At site M, the average age was 53 ± 11, and the age range from 27 to 77. The slight difference in average and distribution of age did not affect the AR studies. As noted, none of these volunteers were suffering from a disease, or had clinical data suggesting the need of medical attention. Bloods were collected using PAXgene Blood RNA tubes, RNA extracted and analyzed using Agilent 44K microarray.

3.2. Identifying the age-reflecting (AR) transcripts

We performed Pearson correlation analyses for the AR transcripts, independently with the H and M datasets. Although members in these two cohorts were not overlapping, we were able to obtain 16 common transcripts that show AR behavior (FDR $\leqslant$ 0.25). The results are shown in Tables 2 and 3, in which transcripts are ordered by the average of the correlation coefficient between the two cohort sites. In Table 2 are listed 11 transcripts whose expression levels are positively correlated with age (AR-increase). The correlation coefficients are 0.505 through 0.347 (FDR: 2.17E-3–2.12E-1) for site H, 0.480 through 0.364 (FDR: 1.06E-1–2.49E-1) for site M. Table 3, on the other hand, lists five transcripts that behave inversely, i.e. their expression levels are negatively correlated with age (AR-decrease). The correlation coefficients were −0.604 through −0.350 (FDR: 3.80E-6–2.08E-1) for site H, −0.518 through −0.364 (FDR: 4.28E-1–2.49E-1) for site M.

Scatter plots of the representative genes, NEFL and CRIP1 (the AR-increase transcripts), and CD248 and SLC4A10 (the AR-decrease transcripts) are shown in Fig. 1. The profiles from sites H and M are nearly superimposable.

To further confirm these findings, we searched the NCBI GEO databases [4] for similar datasets. We found that the GSE23515 data fitted well. Relevant parts of the data are copied in Supplementary

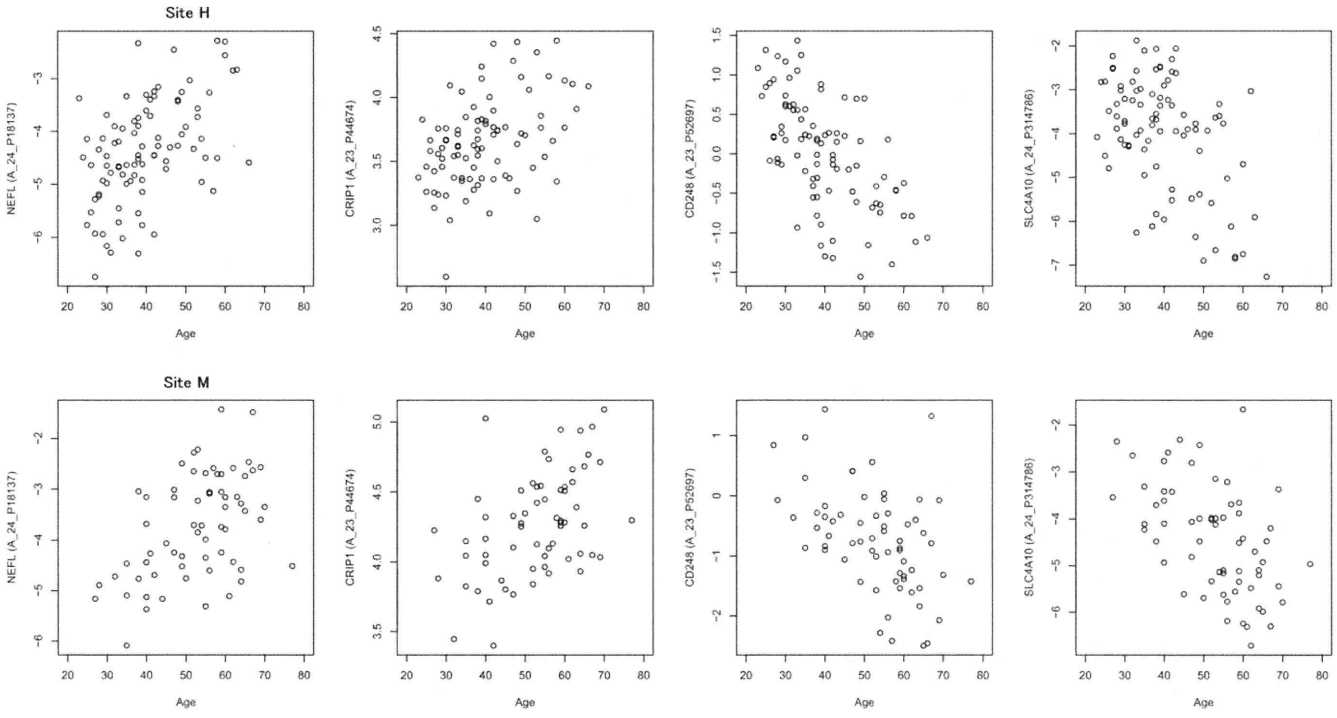

Fig. 1. Representative scatter plots showing correlations between age and some of the AR transcripts. Upper panel shows data from site H, and lower panel data from site M.

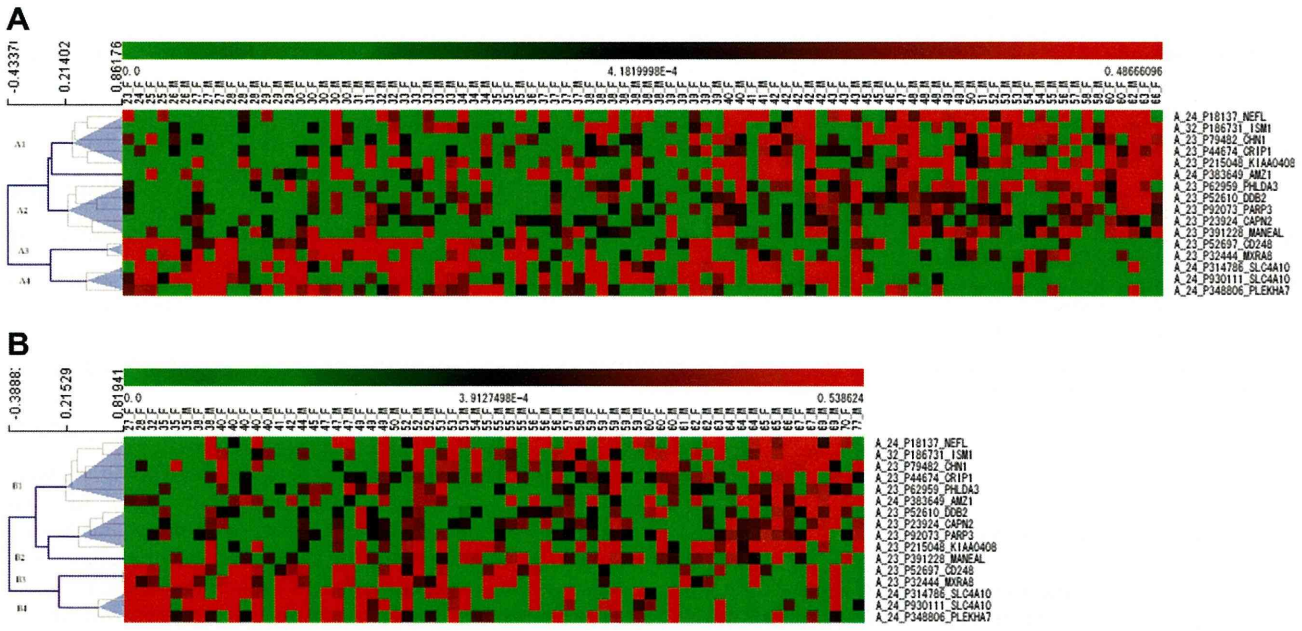

Fig. 2. Hierarchical clustering analysis of 16 AR transcripts based on the expression levels of (A) site H with 90 samples and (B) site M with 64 samples. Details of clustering analyses are described in Materials and methods section. Sample labels represent age and gender.

Tables 1 through 3. Although the subject number is only 24, and the age range is from 21 to 64, the dataset can serve as a perfect source of AR transcripts, and the results showed excellent agreement with ours. For comparison, scattergrams with the four of the transcripts (NEFL, CRIP1, CD248, SLC4A10) are displayed (Supplementary Fig. S1).

3.3. Hierarchical clustering analysis of the AR transcripts

To examine the uniformity/heterogeneity of these and other AR transcripts, we performed hierarchical cluster analysis as shown in Figs. 2A (site H) and 2B (site M). The AR-increase transcripts were grouped into four clusters in Figs. 2A and 2B. Members in cluster A1 show excellent agreement with those in cluster B1. Similarly, members in cluster A2 almost perfectly match with members in clusters B2. As with AR-decrease transcripts, members in A3 and A4 clusters almost perfectly have their counterparts in clusters B3 and B4, respectively. Having these transcripts been extracted independently from the sites H and M, it is highly likely that the AR-increase and AR-decrease transcripts are robust markers, consisting of at least two groups each.

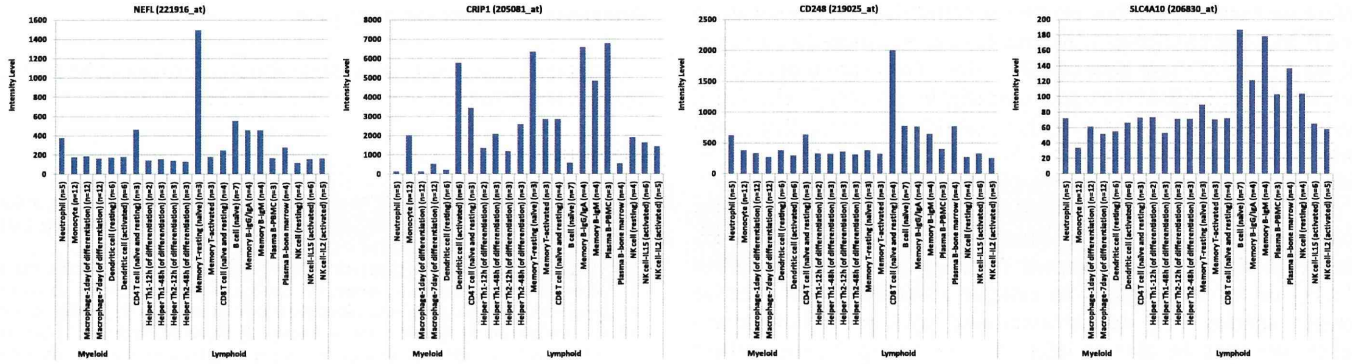

Fig. 3. Expression profiles of the top AR transcripts, NEFL and CRIP1 (AR-increase), and CD248 and SLC4A10 (AR-decrease) in various immune cells under variety of conditions. Texts in parentheses next to the gene symbols indicate Affymetrix probe ID. Data were taken from [4,5].

3.4. Assignment of the AR transcripts to blood immune cell types

Abbas et al. [5] examined gene expression profiles of immune cells under variety of conditions. Although their data were obtained with cultured cells, we took it liberty to refer to these data for assigning our AR transcripts. The selected four AR transcripts in Fig. 1 are displayed in Fig. 3. Other transcripts in Tables 2 and 3 are collectively displayed in Supplementary Fig. S2 in a similar way. The names of referred cells thus obtained are displayed in Tables 2 and 3. Regardless of AR-increase or AR-decrease, significant fractions of the AR transcripts are highly associated with T lymphocytes and B lymphocytes. For example, the top AR-increase transcript, neurofilament light polypeptide (NEFL), is specifically expressed in the memory T resting (naïve) cell, and the top AR-decrease transcript, endosialin (CD248), is highly expressed in the CD8 T (naïve and resting) cell.

3.5. Real-time RT-PCR verification of the AR transcripts

We took CD248, the transcript that showed the strongest correlation with age in microarray expression, and examined it in real-time RT-PCR for verification. We were able to show the expected correlation between this gene expression and age (Fig. 4).

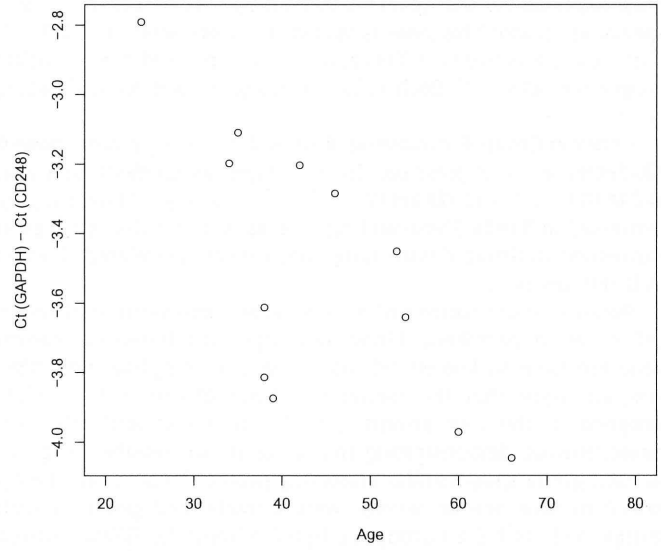

Fig. 4. Real-time RT-PCR verification of the correlation between age and the gene expression level of CD248. Data are shown in scatter plot. The gene expression level of CD248 was normalized to the expression of GAPDH (endogenous control gene).

4. Discussion

Senescence of man and dynamic shift of immune activity have attracted researchers. Studies have been conducted to search for an age-dependent correlation with immune cell differentiation, production, cell population shift in organs or in blood stream [6]. Microarray technologies, which allow transcriptome analyses, have opened the door to a new era in this research field.

We attempted to extract age-reflecting (AR) transcripts in human peripheral blood. The profile of transcripts in the peripheral blood has been believed to be very complex, as population of cells will be unique from a person to person, and will reflect the host's physiological condition, such as disease, infection, injury, fatigue, hormone unbalance and so on, under bewilderingly complex transcriptional controls of immune cells. Nevertheless, we were able to extract AR marker transcripts through cohort studies, independently ran at two sites (90 and 64 participants, age 23–77) using simple correlation analysis between transcripts and age. We found 16 AR transcripts, 11 of which are AR-increase and 5 AR-decrease. Analyses of an another dataset obtained from NCBI GEO have yielded essentially the same results, indicating that these AR transcripts represent a set independent of ethnicity, and can be taken as AR biomarkers of man. Whitney et al. [1] and Eady et al. [7] have attempted similar approaches. Unfortunately, the former authors failed to present a gene list, and the latter authors handled too few samples for qualified data. Furthermore, the latter authors employed isolated peripheral blood mononuclear cells (PBMC), that are liable to errors because of some cell loss, degradation of some mRNA and changes in active genes during purification. As noted, the peripheral blood transcriptome profile is highly affected by donor's physiological condition, such as health condition, exercise, food intake, day rhythm, and many more. Despite these possible factors for complication, our AR transcripts gave rise to reproducible results after taking appropriate precautions.

Most of the genes for AR transcripts are well known, although their roles in AR phenotypes have not been well established. The 16 transcripts do not match the hypothetical longevity genes deduced from SNPs by Sebastiani [8], nor are included in the reference-driven pathway members around sirtuin [9], mTOR [10], Klotho [11]. Though not proved, the 16 AR transcripts may be biomarkers some of which reflect the population change of cells in the blood in the course of aging, and some other reflect gene expression control change within cells. When we examine the 16 transcripts in the GSE22886 dataset in conjunction with the cognate cells, many of them are expressed highly and preferentially in T or B lymphocytes.

Although the roles of the 16 AR marker transcripts in the process of senescence wait further elucidation, we can make some

inference based on the four clusters of transcripts shown in Fig. 2A and B. Members in Group 1 (A1 and B1 clusters) show AR-increase. At least three of these genes (NEFL, CHN1, ISM1) are uniquely expressed (Over 3 IQR of the upper quartile) in memory T cells (Fig. 3 and Supplementary Fig. S2). Notice that the number of these cells shows increase with aging [12]. Thus, genes in this group may reflect changes in memory T cell number in the aging process.

Group 2 consisted of genes in A2 and B2 clusters. These genes also showed AR-increase in expression. DDB2 and PARP3 included in this group are known for their DNA-repair activities [13,14] or for involvement in cellular accumulation of reactive oxygen species [15], deeply associated with premature senescence. We may be able to infer that the Group 2 genes reflect cellular activity change, rather than cell population shift, caused by senescence.

Group 3 consisted of genes in A3 and B3 clusters. These genes showed AR-decrease expression. No definitive AR functions have been reported for the genes in this group, but CD248 may need special attention: This gene is specifically-expressed in CD8 T cell (Fig. 3), and is active [16]. This gene is also expressed in endothelial progenitor cells [17]. Both cells decreased in number with aging [18,19].

Genes in Group 4, consisting of A4 and B4 clusters, also showed AR-decrease in expression. In cell type assignment analysis, SLC4A10 (Fig. 3) and PLEKHA7 (Supplementary Fig. S2) were highly expressed in B cells. These findings suggest that the decline of gene expression in Group 4 with aging may reflect age-related changes in B cell function.

Because of the paucity of data on gene expression in different cell types in peripheral blood, our argument based on known gene functions in known cell, as above, is incomplete. Nevertheless, we argue that the cluster structures of the 16 transcripts assigned to the four groups (Fig. 2) are stable and relatively homogeneous, demonstrating that a significant number of genes in each group show similar clustering profile. Thus, it may be allowed to take one or several well-characterized genes in each group, such as NEFL (Group 1), DDB2 (Group 2), CD248 (Group 3), and SLC4A10 (Group 4), as representing majority of the genes in each of the four groups. We will be able to discover more AR genes as we extend this line of works. As discussed, some of them may reflect dynamic change of immune cell population, and others may represent emerging/recessing function of immune cells. Further studies on the function of these AR genes and their assignment to cells will provide insights into the roles played by immune system in conjunction with aging of man. In addition, this easy assay system could be used for monitoring the health condition of individuals: For example, the stable balance of the marker set may be taken as reflecting a stable health condition, whereas their distorted proportion could be taken as an alarm of physiological change of the examinee. Notice that fatigue, dementia, unnoticed cancer, drug or supplement intake, and many other temporary or long-lasting physiological changes may induce a balance shift.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.018.

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ORIGINAL ARTICLE

Characterization of naturally occurring protease inhibitor-resistance mutations in genotype 1b hepatitis C virus patients

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Abstract
Background and aims Protease inhibitor (PI)-resistant hepatitis C virus (HCV) variants may be present in substantial numbers in PI-untreated patients according to recent reports. However, influence of these viruses in the clinical course of chronic hepatitis C has not been well characterized.
Methods The dominant HCV nonstructural 3 (NS3) amino acid sequences were determined in 261 HCV genotype 1b-infected Japanese patients before pegylated interferon plus ribavirin (PEG-IFN/RBV) therapy, and investigated the patients' clinical characteristics as well as treatment responses including sustained virological response (SVR) rate. HCV-NS3 sequences were also determined in 39 non-SVR patients after completion of the therapy.
Results Four single mutations (T54S, Q80K, I153V, and D168E) known to confer PI resistance were found in 35 of 261 patients (13.4%), and double mutations (I153V plus T54S/D168E) were found in 6 patients (2.3%). Responses to PEG-IFN/RBV therapy did not differ between patients with and without PI-resistance mutations (mutation group, SVR 48%; wild-type group, SVR 40%; $P = 0.38$). On the other hand, two mutations appeared in two non-SVR patients after PEG-IFN/RBV therapy (I153V and E168D, 5.1%).
Conclusions PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. The impact of these mutations in the treatment of PIs is unclear, but clinicians should pay attention to avoid further development of PI resistance.

Keywords HCV · Protease inhibitor · Naturally occurring viral resistance mutations

Introduction

Hepatitis C virus (HCV) infects more than 170 million persons worldwide and thus represents a global health problem. At least 130 million infected individuals are chronic carriers of HCV and are at significant risk of developing liver cirrhosis and hepatocellular carcinoma [1]. The current standard treatment with pegylated interferon plus ribavirin (PEG-IFN/RBV) is complicated by frequent adverse reactions, and a sustained virologic response (SVR) can be achieved only in 50% of patients infected with the most prevalent genotype 1 [2]. In Japan, since 70% of patients are infected with intractable genotype 1b HCV, more effective treatments are urgently required.

A promising approach is the development of specifically targeted antiviral therapies for hepatitis C (STAT-C). HCV-specific protease inhibitors (PIs) target an essential step in HCV replication by blocking the nonstructural 3/4A (NS3/4A) protease-dependent cleavage of the HCV polyprotein

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[1]. Among these NS3/4A PIs, telaprevir, boceprevir, SCH446211, danoprevir (ITMN-191), nalaprevir (SCH900 518), and TMC435 are now under clinical trials [1, 3–7]. In PROVE1 and PROVE2 studies [3, 4] undertaken in North America and Europe, the SVR rate was favorable (67 and 69%, respectively) in a triple therapy regimen including telaprevir. In addition, some studies have suggested that shortening of treatment duration may be possible for patients who achieve a rapid virologic response (RVR) [8, 9].

However the sole use of STAT-C drugs, such as PIs, promotes production and selection of drug-resistant variants in patients experiencing viral rebound during treatment [3, 10, 11] as well as in HCV replicon experiments [11, 12]. Therefore, these drugs should be used in combination with the PEG-IFN/RBV to prevent the appearance of drug-resistant variants. However, Kuntzen et al. [13] demonstrated the presence of these drug-resistant variants in high frequencies (8.6–16.2%) by population-based sequencing in patients not treated with the drugs [1, 13]. Gaudieri et al. [14] have suggested that regions of NS3 protease and NS5B polymerase are likely to be under HLA immune pressure and therapeutic selection, and that drug-resistant variants may occur naturally to escape the immune system. These observations seem quite astonishing and troubling, since a substantial number of patients may not respond to the new therapies such as STAT-C drugs.

In the present study, to assess the prevalence of NS3 mutations conferring PI resistance in HCV genotype 1b-infected Japanese patients who had not been previously treated with PIs, as well as to assess the influence of those mutations in response to PEG-IFN/RBV therapy, the dominant HCV-NS3 sequences were determined in 261 HCV-1b patients before starting the PEG-IFN/RBV therapy.

Methods

Patients

Serum samples were acquired from 261 HCV genotype 1b-infected adult Japanese patients before combination therapy with PEG-IFN (PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2004 and 2008 at the University of Yamanashi, Musashino Red Cross Hospital and Kanazawa University. The therapy was administered according to the standard PEG-IFN/RBV treatment protocol established for Japanese patients by a hepatitis study group of the Ministry of Health, Labor, and Welfare, Japan. Specifically, the patients were subcutaneously administered PEG-IFNα-2b, 1.5 μg/kg body weight, once weekly and RBV 600–800 mg daily per os for 48 weeks. These patients were not infected with human immunodeficiency virus (HIV). The study was approved by the ethics committees of all participating universities and the hospital, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board at Massachusetts General Hospital. Written informed consent was obtained from each study participant.

Amplification and sequencing of full-length HCV genomes

Viral loads were determined using the Amplicor HCV RNA kit, version 2.0 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan test (Roche Diagnostics). HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the manufacturer's protocol. Complementary DNA was synthesised using Superscript II (Invitrogen, Tokyo, Japan) and random primers (Invitrogen), and then amplified by two-step nested PCR using the primers listed in Supplementary Table 1. All samples were initially denatured at 95°C for 7 min, followed by 40 cycles of amplification with denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s using the BD Advantage™ 2 PCR Enzyme system (BD Biosciences Clontech, CA, USA). PCR amplicons were directly sequenced using BigDye Terminator version 3.1 (ABI, Tokyo, Japan) and universal M13 forward/reverse primers using an ABI prism 3130 sequencer (ABI).

Sequence alignment and analysis

Sequences were determined in both directions, particularly for the ambiguous stretches, were assembled using the Vector NTI software (Invitrogen), and base-calling errors were corrected following the inspection of chromatograms. If mixed bases were detected as two different chromatogram peaks at the same residue, only the dominant base was called after evaluation of all overlapping fragments. A consensus sequence was generated from the alignment on the basis of the most common amino acid at each site.

Determination of PI resistance mutations

Multiple viral NS3 mutations were observed in amino acid positions reported to confer PI resistance among 261 patients: V36, Q41, F43, T54, V55, Q80, R109, I153, R155, A156, D168, V170, and M175. NS3 amino acid mutations with proven PI resistance in previously published studies (Table 1) were designated as resistance proven mutations (e.g., V36M/A). Mutations in the PI-resistance site not known to confer drug resistance were designated resistance unproven mutations (e.g., V36I). Patients were allocated to two groups according to the presence of PI-resistance

mutations (including resistance unproven mutations), and clinical characteristics including HCV RNA levels and responses to PEG-IFN/RBV therapy were compared. To assess the influence of PEG-IFN/RBV therapy on NS3 mutational status, posttreatment HCV-NS3 sequences in 39 of 58 non-SVR patients were also examined.

Statistical analysis

Statistical differences in the data, including all available patients' demographic, biochemic, hematologic, and virologic data such as sequence variation factors, were determined among the various groups by Student's *t* test or Mann–Whitney *U* test for numerical variables and Fisher's exact probability test for categorical variables.

Results

Prevalence of dominant PI-resistance-associated nonstructural 3 mutations in untreated patients

Figure 1 shows the frequency of substitutions in 261 patients for each of 181 NS3 protease amino acid residues compared to the consensus sequence. A total of 41 resistance proven mutations were detected in 35 (13.4%) patients: T54S (14 patients, 5.4%), Q80K (1 patient, 0.4%), I153V (22 patients, 8.4%), D168E (4 patients, 1.5%), T54S plus I153V double mutation (4 patients, 1.5%), and I153V plus D168E double mutation (2 patients, 0.8%). The mutation number increased to 54 in 47 (18.0%) patients when resistance unproven mutations were included: V36I (2 patients, 0.8%), I153L (11 patients, 4.2%), and I153V plus V36I double mutation (2 patients, 1.5%). Double mutations were found in 7 patients (2.7%) (Table 1). Q80L was observed in 47 (18%) patients but these were excluded from consideration because a previous study demonstrated that this mutation does not confer resistance [15]. All mutations observed in this study would confer low- to moderate-level PI resistance according to previous studies [6, 15–19]. No mutations conferring high-level resistance such as R155 or A156 [11, 17, 19–22] were observed.

Clinical characteristics of patients with PI-resistance mutations

Table 2 presents the characteristics of patients classified according to the presence of PI-resistance mutations

Table 1 Prevalence of PI-resistance-associated NS3 mutations

Drug-resistance mutations described in the literature				Detected resistance mutations
NS3 residue	Resistance mutations	Drugs	References	Genotype 1b ($N = 261$), (%)
V36	A, M, L, G, C	Telaprevir, Boceprevir	[1, 3, 4, 10, 11, 19, 31, 37]	I × 2 (0.8)
Q41	R	ITMN-191, Boceprevir	[19]	
F43	S, C	ITMN-191, Boceprevir, Telaprevir, TMC435	[15, 19]	
T54	A, S	Telaprevir, Boceprevir, SCH900518	[1, 3, 10, 11, 19, 20, 31, 38]	**S × 14 (5.4)**
V55	A	Boceprevir	[1]	
Q80	R, K	TMC435	[6, 15]	**K × 1 (0.4)**
R109	K	SCH446211	[17]	
I153	V	SCH446211	[17]	**V × 22 (8.4)**, L ×11 (4.2)
R155	K, T, I, M, G, L, S, Q	Telaprevir, Boceprevir, ITMN-191, BILN2061, TMC435	[1, 3, 4, 6, 10, 11, 15, 19, 20]	
A156	S, T, V, I, G	Telaprevir, Boceprevir, ITMN-191, BILN2061, SCH446211, TMC435, SCH900518	[1, 3, 4, 10, 11, 15, 17, 19, 20, 38]	
D168	A, V, E, N, T, H	BILN2061, ITMN-191, TMC435	[6, 15, 20]	**E × 4 (1.5)**
V170	A	Telaprevir, Boceprevir	[1, 19, 20]	
M175	L	Boceprevir	[39]	
Total number (%) of patients with resistance proven mutations				35 (13.4)
Total number (%) of patients with resistance proven and unproven mutations				47 (18.0)

Amino acid mutations conferring PI resistance in the literatures and those observed in PI-treatment-naive patients in this study are indicated. Bold indicates resistance proven mutations, and the others indicate resistance unproven mutations

Double mutations found were as follows: V36I and I153V × 1, T54S and I153V × 4, I153V and D168E × 2

(including resistance unproven mutations). Age, sex ratio, body mass index, alanine aminotransferase (ALT) levels, serum albumin, platelet count, and fibrosis stage did not differ between the NS3 mutation and wild-type groups. No significant difference was observed between the two groups in the parameters of PEG-IFN/RBV treatment response, HCV sequence variations in interferon sensitivity determining region (ISDR), Core 70, interferon plus ribavirin resistance-determining region (IRRDR), or interleukin 28B (IL28B) single nucleotide polymorphism (SNP) (rs8099917; T/G and G/G vs. T/T) [23–30]. These clinical variables were also compared between the mutation group defined as resistance proven mutations and the wild-type group, but no notable differences were observed.

Unimpaired in vivo fitness of viral strains with resistance mutations

Because most PI-resistance mutations described till date have been associated with reduced replicative capacity of varying degrees [1, 10, 11, 13, 17, 20–22, 31, 32], we examined viral replication levels in patients with drug-resistance mutations (Fig. 2). The estimated P value indicated no significant difference between the mutation (median 1,500 KIU/ml) and wild-type (median 1,800 KIU/ml) groups ($P = 0.69$). The results indicate that drug-resistant HCVs were not necessarily impaired in their ability to replicate in vivo. However, patients with double mutations ($N = 7$) tended to have low viral loads (median 1,200 KIU/ml) ($P = 0.09$).

Resistance mutations and virologic response to PEG-IFN/RBV therapy

To determine the difference in virologic response to PEG-IFN/RBV therapy according to the PI mutation, frequency of HCV RNA levels below detection at 4 weeks (rapid viral response, RVR) and 12 weeks (complete early viral response, cEVR), and SVR rate (%) were investigated in each group. The frequency of HCV RNA levels below detection at 4 and 12 weeks was 14 and 50%, respectively, in the mutation group, and was 11 and 46%, respectively, in the wild-type group. The SVR rate was 48 and 40% in the mutation and wild-type groups, respectively ($P = 0.38$). No significant difference was observed between the two groups in any of the indexes investigated (Table 2). The time-dependent viral clearance rate during PEG-IFN/RBV therapy was estimated in 133 patients including 25 patients (19%) with PI-resistance mutations available for the analysis. Kaplan–Meier analysis demonstrated that HCV clearance did not differ between the two groups with and without resistance mutations (log-rank test, $P = 0.30$) (Fig. 3).

Changes in nonstructural 3 amino acid sequence diversity during PEG-IFN/RBV therapy

Full-length NS3 protease sequences were determined in 39 non-SVR patients after PEG-IFN/RBV therapy. A single amino acid change at resistance-associated sites in two patients was observed. In one patient, isoleucine (Ile) at position 153 changed to valine (Val), and glutamic acid (Glu) changed to aspartic acid (Asp) at position 168 in the second (Fig. 4). At the nucleotide level, ATC (Ile) changed to GTC (Val) in I153V, and GAA (Glu) changed to GAC (Asp) in E168D. Both mutations were caused by one nucleotide exchange. No other changes were observed in the other 37 patients.

Discussion

Here we report that in 18% (47/261) HCV genotype 1b-infected patients who had not been previously treated with NS3 PIs, the viral genome contained dominant amino acid mutations within the NS3 PI-resistance sites. Even after confining the data to established PI-resistance mutations, the mutation rate was still significant in 13.4% (35/261). No clinical differences were observed between patients

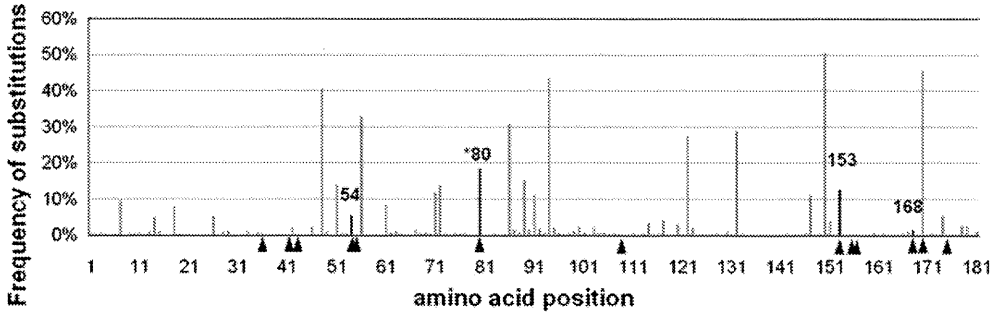

Fig. 1 Frequency of polymorphic mutations for each of the 181 NS3 protease amino acid residues in 261 patients. *Arrowheads* indicate the sites reported to confer PI resistance. *Dark bars* denote the amino acid variations at the resistant sites in this study. **80*, we detected one resistant mutation (Q80K) and 47 (18%) non-resistant variations (Q80L) at the 80th residue

Table 2 Characteristics of patients with or without HCV genomes harboring drug-resistance mutations

Characteristics	Mutation type ($N = 47$)	Wild-type ($N = 214$)	P value
Patients' characteristics			
Age, median (range)	59 (46–72)	57 (19–77)	0.17
Male, no. (%)	26 (55)	112 (52)	0.70
BMI, median (range)	23.2 (15.5–31.9)	22.8 (16.1–31.9)	0.41
ALT IU/ml	81.3 ± 72.6[a]	74.8 ± 51.9	0.93
Serum albumin g/dl	4.00 ± 0.37	4.01 ± 0.36	0.81
Platelet count × 10^4/μl	15.8 ± 4.3	14.5 ± 4.8	0.18
HCV RNA KIU/ml, median (range)	1,500 (58–6,310)	1800 (28–15,849)	0.69
Fibrosis, no. (%)			0.97
F0	0 (0)	7 (3)	
F1	23 (50)	89 (42)	
F2	9 (20)	52 (24)	
F3	9 (20)	40 (19)	
F4	5 (11)	26 (12)	
IFN pre-treatment no. (%)	15/40 (38)[b]	66/172 (38)	1.00
IL28B (rs8099917) T/G or G/G no. (%)	6/20 (30)	19/67 (28)	1.00
Response to PEG-IFN/RBV therapy			
SVR total cases no. (%)	22/46 (48)	83/210 (40)	0.38
RVR in total cases no. (%)	6/44 (14)	22/195 (11)	0.83
cEVR in total cases no.(%)	22/44 (50)	92/200 (46)	0.75
SVR 48w treatment no. (%)	16/29 (55)	55/130 (42)	0.29
End of treatment response no. (%)	26/41 (63)	123/202 (61)	0.91
HCV genome sequence variation			
ISDR mutation ≤1 no. (%)	32/46 (70)	167/210 (80)	0.21
Core70 R no. (%)	26/44 (59)	136/210 (65)	0.56
IRRDR mutation >3 no. (%)	25/38 (66)	107/190 (56)	0.34

[a] Mean ± SD

[b] Number/total number (%)

harboring viruses with and without these mutations. Moreover, no differences were observed in the responses of either group to PEG-IFN/RBV therapy.

Recent studies reported that significant number of patients who were never treated with PI possess viral sequences with PI-resistance-associated NS3 mutations. In these studies, the prevalence of PI-resistance mutations was determined to be 8.6–16.2% [13, 14], in HCV genotype 1- and 3-infected patients in European–American populations. These patients were often coinfected with HIV. Analysis of the public HCV databases (EuHCVdb and Los Alamos) also reported the presence of naturally occurring PI-resistance-associated NS3 mutations in worldwide isolates [33]. However, in vivo and in vitro studies demonstrated that most of the mutations observed conferred only low- to moderate-level PI resistance [7, 13, 14, 34, 35]. Regarding viral fitness, PI-resistant HCVs show lower fitness at varying degrees as revealed by in vitro studies [1, 10, 11, 17, 20–22, 31, 32], but HCV RNA levels in a clinical study did not differ significantly. The response to PEG-IFN/RBV therapy was almost comparable to that in HCV-infected patients without PI-resistance mutations either in HCV replicon experiments or in a clinical study of small number of treated patients [34].

The prevalence of 13.4% for PI-resistance-proven patients observed in the present study was almost comparable to the results of previous studies. Although HIV is known to increase HCV replication in coinfection with HCV [36], and HIV patients are often treated with the HIV-specific PIs, the HIV infection might not affect the natural occurrence of HCV-specific PI-resistance mutations since our studied patients were all proven to be free from coinfection with HIV infection. As shown in Table 1 and Fig. 1, I153 V (22/261, 8.4%), T54S (14/261, 5.4%), and D168E (4/261, 1.5%) were among the most prevalent PI-resistance-proven mutations in the present study. The most frequent mutation detected in our study I153V was reported to appear secondarily to the occurrence of R109K mutations in a HCV replicon system [17]. Although the role of this mutation is not understood, the I153V mutation on its own conferred SCH446211 resistance to the HCV replicon to a lesser degree [17]. Interestingly, I153V was often found in double mutations in our study, as shown in Fig. 2. This suggests analogy between in vitro and in vivo data. T54S and D168E, the other frequent mutations, have been also reported to occur as single dominant mutations in previous in vitro or in vivo studies in HCV genotype 1

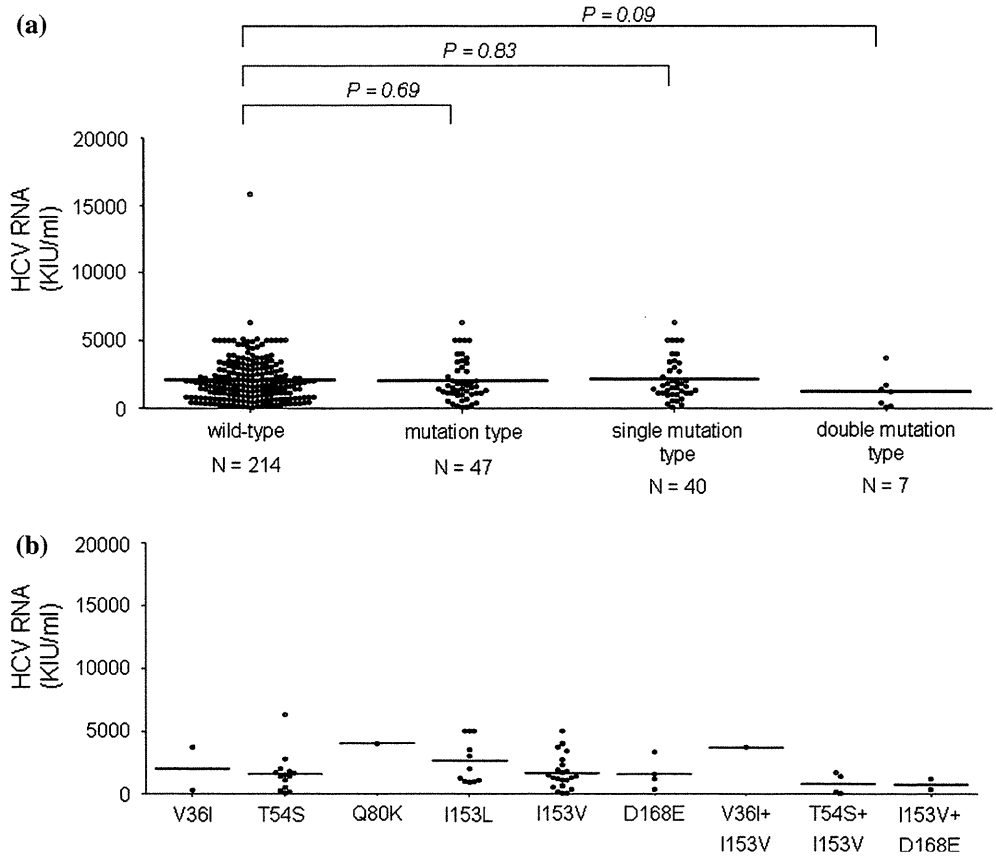

Fig. 2 In vivo fitness of HCV with PI-resistance-associated NS3 mutations. HCV RNA levels were compared between patients with and without NS3 PI-resistance-associated mutations (**a**) and between patients with each resistance mutation (**b**). The estimated *P* value (Mann–Whitney *U* test) indicates no significant difference between the wild-type and other groups (wild-type vs. mutation type, wild-type vs. single mutation type, and wild-type vs. double mutation type). (Wild-type, $N = 214$; mutation type, $N = 47$; single mutation type, $N = 40$; double mutation type, $N = 7$; V36I, $N = 2$; T54S, $N = 14$; Q80K, $N = 1$; I153L, $N = 11$; I153V, $N = 22$; D168E, $N = 4$; E176A, $N = 1$; V36I + I153V, $N = 1$; T54S + I153V, $N = 4$, and I153V + D168E, $N = 2$)

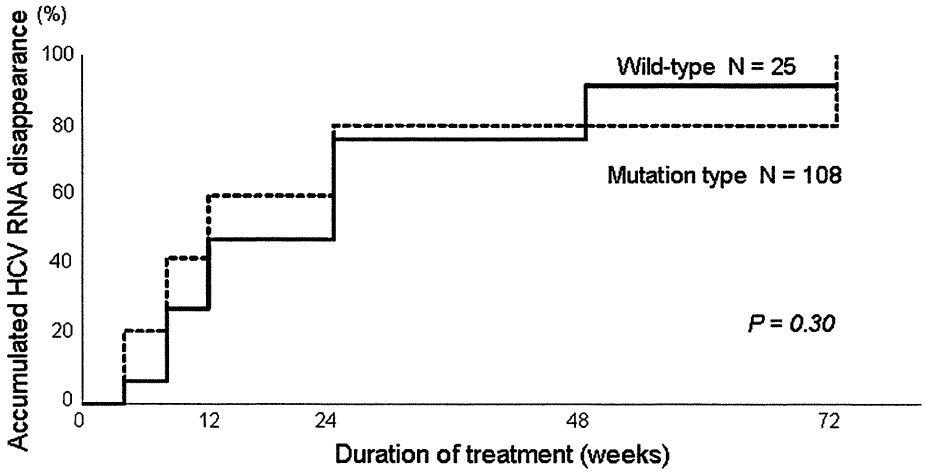

Fig. 3 Comparison of virologic response to PEG-IFN/RBV therapy between HCV-infected patients with and without PI-resistance-associated NS3 mutations. Time-dependent HCV clearance rate analysis was based on serum HCV RNA positivity during PEG-IFN/RBV therapy for HCV isolates with resistance mutations or wild-type sequences. A total of 133 patients for whom the limit of viral genome detection could be determined were analyzed. Among this group, NS3 mutations were detected in 25 patients (19%). The estimated *P* value (log-rank test) shows no significant difference between the two groups ($P = 0.30$)

infections showing moderate degrees of resistance [16, 18, 19].

Most PI-resistance mutations described to date have been associated with varying degrees of reduced replicative capacity [10, 11, 17, 20–22, 31, 32]. In the present study, HCV RNA levels of those patients with low- to moderate-level resistance mutations were similar to those in patients in the wild-type groups, suggesting that in vitro viral fitness

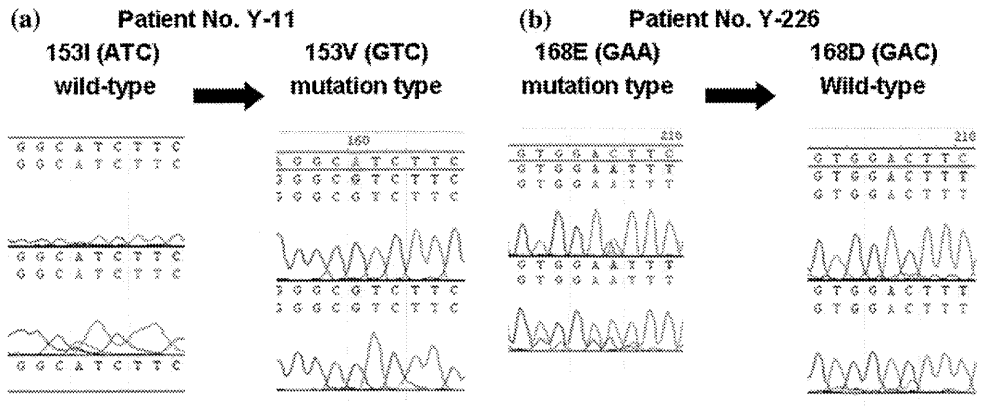

Fig. 4 Appearance of PI-resistance-associated NS3 mutations during the PEG-IFN/RBV therapy. Chromatograms show part of the HCV NS3 sequence demonstrating PI-resistance mutations in two patients receiving therapy. **a** Site 153 isoleucine (Ile) (ATC) changed to valine (Val) (GTC), **b** Site 168 glutamic acid (Glu) (GAA) changed to aspartic acid (Asp) (GAC)

does not necessarily reflect in vivo viral fitness. This, however, does not rule out the possibility that some unknown compensatory viral mutations might have resulted in upregulation of reduced viral fitness. Interestingly, although the replicative capacity conferred by a single mutation seemed to be the same, the HCV RNA levels of double mutations were frequently low, suggesting that double mutations might weaken viral fitness.

In previous studies, clinical characteristics representing the state of liver disease other than HCV RNA levels were not studied in patients with PI-resistance mutations. In this study, we show that those clinical characteristics did not differ according to the presence of viral NS3 mutations. As shown in Table 2, age, sex ratio, fibrosis stage, ALT levels, serum albumin, platelet count, and past history of IFN pretreatment did not differ according to the presence of NS3 mutations. These results suggest that NS3 mutations occur independently of disease progression. Moreover, no evident differences were observed between viral and host factors known to affect IFN-based treatment responses. However, viral amino acid variations in the core and NS5A or the allelic frequency of IL28B SNPs, which were recently reported for the close relationship of responses to PEG-IFN/RBV therapy, did not differ between the two groups.

A significant outcome of the present study is the demonstration that PI-resistance mutations might not affect responses to PEG-IFN/RBV therapy. Previous in vitro studies demonstrated that HCV replicons harboring PI-resistance mutations were also sensitive to IFN treatment [31]. In addition, recent clinical studies also indicated that PI-resistance mutations were sensitive to the PEG-IFN/RBV [10, 34]. However, our analysis was more comprehensive because viral and host factors that contribute to treatment responses were simultaneously analyzed. A unique aspect of the present study is that we investigated the influence of the PEG-IFN/RBV treatment on the occurrence of new PI mutations by direct nucleotide sequencing, and were able to show that the PEG-IFN/RBV might not induce amino acid mutations.

Will the pre-existence of naturally occurring PI-resistance mutations have an influence on future treatment of HCV infections? Since new PIs are on the verge of clinical use, all clinicians should bear in mind the substantial numbers of HCV-infected patients with PI-resistance mutations. Although the degree of resistance is considered to be low or moderate in untreated patients, weak resistance might progress to more potent resistance with additional mutations, when PIs become widely used. Therefore, all clinicians need to be sufficiently prepared for the possibility of later onset of PI-resistance mutations that confer greater drug resistance and concomitant poorer responses to therapy. In SPRINT-1 study, the lead-in therapy was associated with a modestly lower rate of breakthrough than with no lead in [7]. Considering that PEG-IFN/RBV was equally effective for PI-resistant viruses, sufficient "lead-in" therapy before the administration of PIs could be an option in the forthcoming triple therapy modality.

In conclusion, we demonstrate here that PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. Although the degree of resistance might not be strong, clinicians will need to consider this upon the introduction of triple therapy.

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Comparative Analysis of Various Tumor-Associated Antigen-Specific T-Cell Responses in Patients with Hepatocellular Carcinoma

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Many tumor-associated antigens (TAAs) recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials. However, there are very few in the field of immunotherapy for hepatocellular carcinoma (HCC) because there have not been comparative data regarding CTL responses to various TAAs. In the present study, using 27 peptides derived from 14 different TAAs, we performed comparative analysis of various TAA-specific T-cell responses in 31 HCC patients to select useful antigens for immunotherapy and examined the factors that affect the immune responses to determine a strategy for more effective therapy. Twenty-four of 31 (77.4%) HCC patients showed positive responses to at least one TAA-derived peptide in enzyme-linked immunospot assay. The TAAs consisting of cyclophilin B, squamous cell carcinoma antigen recognized by T cells (SART) 2, SART3, p53, multidrug resistance-associated protein (MRP) 3, alpha-fetoprotein (AFP) and human telomerase reverse transcriptase (hTERT) were frequently recognized by T cells and these TAA-derived peptides were capable of generating peptide-specific CTLs in HCC patients, which suggested that these TAAs are immunogenic. HCC treatments enhanced TAA-specific immune responses with an increased number of memory T cells and induced *de novo* T-cell responses to lymphocyte-specific protein tyrosine kinase, human epidermal growth factor receptor type 2, p53, and hTERT. Blocking cytotoxic T-lymphocyte antigen-4 (CTLA-4) resulted in unmasking of TAA-specific immune responses by changing cytokine and chemokine profiles of peripheral blood mononuclear cells stimulated by TAA-derived peptides. *Conclusion:* Cyclophilin B, SART2, SART3, p53, MRP3, AFP, and hTERT were immunogenic targets for HCC immunotherapy. TAA-specific immunotherapy combined with HCC treatments and anti-CTLA-4 antibody has the possibility to produce stronger tumor-specific immune responses. (HEPATOLOGY 2011;53:1206-1216)

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and becoming an important public health concern.[1,2] Although many kinds of treatments have been performed for HCC, their effects are limited because the recurrence rate of HCC is very high; therefore, the development of new therapeutic options to prevent recurrence is necessary.[3,4]

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Many tumor-associated antigens (TAAs) and their epitopes recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials for several cancers.[5-21] The epitopes have been under investigation for the treatment of cancer, with major clinical responses in some trials.[22,23] With regard to immunotherapy for HCC, few kinds of TAAs and their epitopes have been used and only clinical data of α-fetoprotein (AFP) have been reported.[24,25] In human trials targeting AFP, it is possible to raise an AFP-specific T-cell response using AFP-derived peptides, but this has shown little

Abbreviations: AFP, alpha-fetoprotein; CTL, cytotoxic T cell; ELISPOT, enzyme-linked immunospot; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen; hTERT, human telomerase reverse transcriptase; IFN, interferon; Lck, lymphocyte-specific protein tyrosine kinase; MRP, multidrug resistance-associated protein; PBMC, peripheral blood mononuclear cell; TAA, tumor-associated antigen.

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antitumor effect. On the other hand, immunotherapy trials using autologous tumor lysate or dendritic cells have shown statistically significant improvements in the risk of HCC recurrence and recurrence-free survival.[26] These reports suggest that tumor antigen-specific immunotherapy is effective to reduce the recurrence rate after HCC treatment; therefore, it is necessary to find immunogenic antigens or their epitopes to develop more effective immunotherapy.

In addition, in the field of molecular targeting therapies, developments of monoclonal antibodies targeting immunomodulatory molecules to enhance antitumor immunity are progressing and some of these are under clinical trial.[27] In particular, clinical data of anti-cytotoxic T-lymphocyte antigen-4 (anti-CTLA-4) antibody have shown durable objective response and stable disease in melanoma patients.[28]

In the present study we performed comparative analysis of various TAA-specific T-cell responses in patients with HCC and examined the factors that affect the immune responses, including anti-CTLA-4 antibody. This approach offers useful information to select immunogenic TAAs and to develop a new strategy for HCC immunotherapy.

Patients and Methods

Patients and Laboratory Testing. In this study we examined 31 human leukocyte antigen (HLA)-A24-positive patients with HCC, 29 chronic hepatitis C patients without HCC, who were diagnosed by liver biopsy, and 11 healthy blood donors who did not have a history of cancer and were negative for hepatitis B surface antigen and anti-hepatitis C virus (HCV) antibody (Ab). The diagnosis of HCC was histologically confirmed in 21 patients. For the remaining 10 patients the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic computed tomography (CT).[29]

HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal blood donors was performed as described.[19] The pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.[30] The severity of liver disease was evaluated according to the criteria of Desmet et al.[31] using biopsy specimens of liver tissue.

All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University, No. 829).

Table 1. Peptides

Peptide No.	Peptide Name	Source	Reference	Amino Acid Sequence	Number of Specific Spots in Normal Donors (Mean SD)
1	$ART1_{188}$	ART1	5	EYCLKFTKL	0.9 ± 1.1
2	$ART4_{161}$	ART4	6	AFLRHAAL	0.3 ± 0.5
3	$ART4_{899}$	ART4	6	DYPSLSATDI	0.6 ± 1.0
4	$Cyp\text{-}B_{109}$	Cyp-B	7	KFHRVIKDF	0.5 ± 0.9
5	$Cyp\text{-}B_{315}$	Cyp-B	7	DFMIQGGDF	1.2 ± 1.7
6	Lck_{208}	Lck	8	HYTNASDGL	0.3 ± 0.6
7	Lck_{486}	Lck	8	TFDYLRSVL	0.2 ± 0.8
8	Lck_{488}	Lck	8	DYLRSVLEDF	0.9 ± 1.5
9	$MAGE1_{135}$	MAGE-A1	9	NYKHCFPEI	1.0 ± 0.9
10	$MAGE3_{195}$	MAGE-A3	10	IMPKAGLLI	1.4 ± 1.7
11	$SART1_{1690}$	SART1	11	EYRGFTQDF	0.9 ± 1.3
12	$SART2_{899}$	SART2	12	SYTRLFLIL	1.0 ± 1.4
13	$SART3_{109}$	SART3	13	VYDYNCHVDL	2.1 ± 1.9
14	$Her\text{-}2/neu_8$	Her-2/neu	14	RWGLLLALL	1.4 ± 2.0
15	$p53_{125}$	p53	15	TYSPALNKMF	1.4 ± 1.5
16	$p53_{161}$	p53	16	AIYKQSQHM	0.4 ± 0.6
17	$p53_{204}$	p53	17	EYLDDRNTF	1.1 ± 1.5
18	$p53_{211}$	p53	17	TFRHSVVV	0.9 ± 1.9
19	$p53_{235}$	p53	17	NYMCNSSCM	2.1 ± 2.6
20	$MRP3_{503}$	MRP3	18	LYAWEPSFL	0.2 ± 0.5
21	$MRP3_{692}$	MRP3	18	AYVPQQAWI	1.5 ± 2.1
22	$MRP3_{765}$	MRP3	18	VYSDADIFL	0.9 ± 1.0
23	AFP_{357}	AFP	19	EYSRRHPQL	1.8 ± 2.0
24	AFP_{403}	AFP	19	KYIQESQAL	1.1 ± 1.5
25	AFP_{434}	AFP	19	AYTKKAPQL	0.8 ± 1.1
26	$hTERT_{167}$	hTERT	20	AYQVCGPPL	0.8 ± 1.1
27	$hTERT_{324}$	hTERT	20	VYAETKHFL	0.5 ± 0.7
28	HIV env_{584}	HIV env	32	RYLRDQQLL	1.3 ± 2.0
29	HCV $NS3_{1031}$	HCV NS3	33	AYSQQTRGL	ND
30	CMV $pp65_{328}$	CMV pp65	34	QYDPVAALF	13.3 ± 15.7

ND, not determined.

Peptides, Cell Lines, and Preparation of PBMCs. Twenty-seven peptides derived from 14 different TAAs (Table 1), human immunodeficiency virus (HIV) envelope-derived peptide ($HIVenv_{584}$),[32] HCV NS3-derived peptide ($HCVNS3_{1031}$),[33] and cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$),[34] which were identified as HLA-A24 restricted CTL epitopes in previous studies, were used. Peptides were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry and their purities were determined to be >80% by analytical high-performance liquid chromatography (HPLC). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 500 μg/mL hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS.[35] PBMCs were isolated before HCC treatments as described.[20] In 12 patients their PBMCs were also obtained 4 weeks after treatments.

Table 2. Characteristics of the Patients Studied

Clinical Diagnosis	No. of Patients	Sex M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/ml) Mean ± SD	Child Pugh (A/B/C)	Diff. Degree* (wel/mod/ por/ND)	Tumor Size† (large/small)	Tumor Multiplicity (multiple/solitary)	Vascular Invasion (+/−)	TNM Stage (I/II/IIIA/IIIB/ IIIC/IV)
Normaldonors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND
Chronichepatitis	29	16/13	59 ± 10	92 ± 94	31 ± 87	27/2/0	ND	ND	ND	ND	ND
HCC	31	23/8	71 ± 4	74 ± 33	1768 ± 9103	20/10/1	11/10/0/10	22/9	20/11	9/22	10/12/3/1/2/3

*Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
†Tumor size was divided into either "small" (≦2 cm) or "large" (>2 cm).

CTL Induction and Cytotoxicity Assay. CTL induction and cytotoxicity assays were performed as described.[20] Briefly, stimulated PBMCs were added at effector to target ratios of 100:1, 50:1, 25:1, 13:1, 6:1, and 3:1. In cases where the number of CTLs was insufficient, cytotoxicity assays were performed at effector to target ratios less than 100:1.

Interferon Gamma IFN-γ Enzyme-Linked Immunospot (ELISPOT) Assay. IFN-γ ELISPOT assays were performed as reported.[20] Responses to TAA-derived peptides were considered positive if more than 10 specific spots were detected, which is greater than the mean plus 3 standard deviations (SDs) of the baseline response detected in 11 normal blood donors (Table 1), and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Responses to HIV-, HCV-, and CMV-derived peptides were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. In ELISPOT assay with blocking CTLA-4, anti-human CTLA-4 (eBioscience, Tokyo, Japan) was added at a final concentration of 50 μg/mL, which has been described to have maximum effect in *in vitro* cultures.[36] As a control, functional grade mouse immunoglobulin G (IgG)2a isotype control was used. The assay with blocking CTLA-4 was performed in triplicate and the results were statistically analyzed using the unpaired Student's *t* test.

Cytokine and Chemokine Profiling. The effect of CTLA-4 antibody on TAA-specific T-cell responses was also analyzed by cytokine and chemokine profiling. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay (Bio-Rad, Hercules, CA). These included interleukin (IL)-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, RANTES, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF). Eight standards (ranging from 2 to 32,000 pg/mL) were used to generate calibration curves for each cytokine. Data acquisition and analysis were carried out using Bio-plex Manager software v. 4.1.1.

Cytokine Secretion Assay. TAA-specific IFN-γ-producing T cells were also analyzed by cytokine secretion assay. The assay was performed with the MACS cytokine secretion assay (Miltenyi Biotec K.K., Tokyo, Japan), in accordance with the manufacturer's instructions. Briefly, 5,000,000 PBMCs were pulsed with TAA-derived peptides for 16 hours and then incubated with 20 μL of IFN-γ detection antibody, 10 μL of anti-CD8-APC Ab (Becton Dickinson, Tokyo, Japan), 10 μL of anti-CCR7-FITC Ab (eBioscience, Tokyo, Japan), and 10 μL of anti-CD45RA-PerCP-Cy5.5 Ab (eBioscience, Tokyo, Japan) for 10 minutes at 4°C. After washing with a cold buffer (phosphate-buffered saline/0.5% bovine serum albumin with 2 mM EDTA), the cells were resuspended with 500 μL of cold buffer and analyzed using FACSCalibur (Becton Dickinson, Tokyo, Japan). As a positive control, CMVpp65$_{328}$-specific IFN-γ-producing T cells were also analyzed by the same methods. The number of IFN-γ-producing T cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs.

Results

Patient Profile. The clinical profiles of the 11 healthy blood donors, 29 patients with chronic hepatitis C, and 31 patients with HCV-related HCC analyzed in the present study are shown in Table 2 and Fig. 1. Using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (6th v.), 10, 12, 3, 1, 2, and 3 patients were classified as having stage I, II, IIIA, IIIB, IIIC, and IV tumors, respectively.

Detection of TAA-Specific T Cells in HCC Patients. First we examined the frequency of cells that specifically reacted with TAA-derived and control peptides in HCC patients. Fifty-one responses in total were observed against TAA-derived peptides. Twenty-

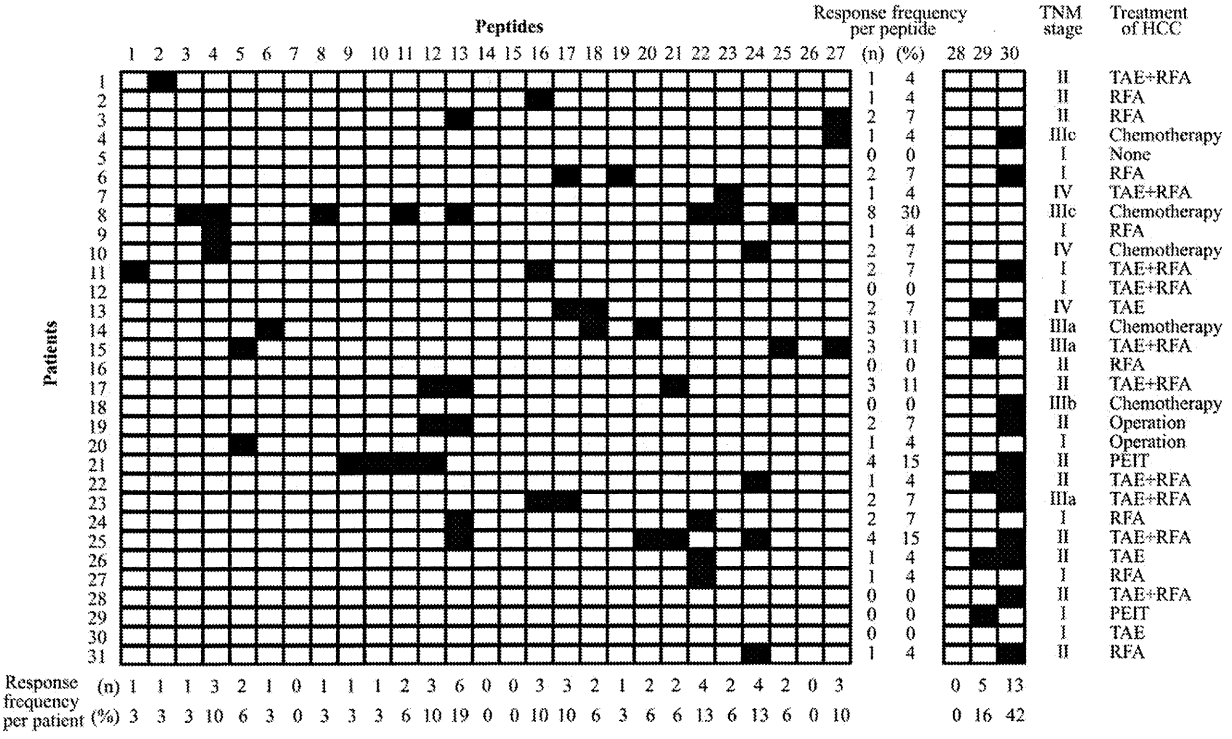

Fig. 1. TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses. Results of all HCC patients examined are shown. The T-cell responses were examined by IFN-γ ELISPOT assay. Responses to peptides were considered positive if more than 10 specific spots per 300,000 PBMCs were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Black boxes indicate the presence of a significant IFN-γ T-cell response to peptides. Peptide sequences are described in Table 1 and characteristics of patients in Table 2.

four of 31 (77.4%) patients showed positive responses to at least one TAA-derived peptide and most of them showed responses to 1 to 4 kinds of TAA-derived peptide. Twenty-three of 27 (85.2%) TAA-derived peptides were recognized by T cells of at least one patient. Peptides 4, 12, 13, 16, 17, 22, 24, and 27 were recognized in more than two patients, suggesting that these peptides were immunogenic. Peptides 28 (HIV env_{584}), 29 (HCV $_{1031}$), and 30 (CMV $pp65_{328}$) were recognized by 0 (0%), 5 (16%), and 13 (42%) patients, respectively.

The magnitude of TAA-specific T-cell responses was assessed by the frequency of peptide-specific IFN-γ-producing T cells in the PBMC population (Fig. 2A). The range of TAA-derived peptide-specific T-cell frequency was 10-60.5 cells/300,000 PBMCs. Those specific to peptides 13 and 16 numbered more than 30 cells/300,000 PBMCs, suggesting that these peptides were immunogenic. The frequencies of T cells specific to HCV- and CMV-derived peptides were 12-22 cells and 12-92/300,000 PBMCs, respectively.

Whether these TAA-derived peptides were capable of generating peptide-specific CTLs from PBMCs was investigated in HCC patients. The seven peptides were selected according to the magnitude of TAA-specific T-cell responses determined by the frequency of T cells with a positive response. The CTLs generated with these peptides were cytotoxic to C1RA24 cells pulsed with the corresponding peptides (Fig. 2B).

Comparison of TAA-Specific T-Cell Responses Between the Patient Groups With and Without HCC. To characterize the immunogenicity and specificity of TAA-derived peptides, we compared T-cell responses to the peptides derived from TAA, HIV, HCV, and CMV among three groups consisting of normal blood donors, patients with chronic hepatitis C, and patients with HCV-related HCC. A significant TAA-specific T-cell response was not detected in normal blood donors (Fig. 3A). A response was detected in both chronic hepatitis C and HCC patient groups, but it was more frequently observed in HCC patients. HIV-specific T-cell response was not detected in any group. HCV-specific T-cell response rate was not different between the groups with chronic hepatitis C and HCC. CMV-specific T-cell response rates were similar among the three groups. Similar tendencies were observed in the analysis of individual peptides (Fig. 3B). We also examined the frequency of T cells responsive to peptides among the three groups. The mean frequency of TAA-specific T cells without *in vitro* expansion was higher in HCC patients than in

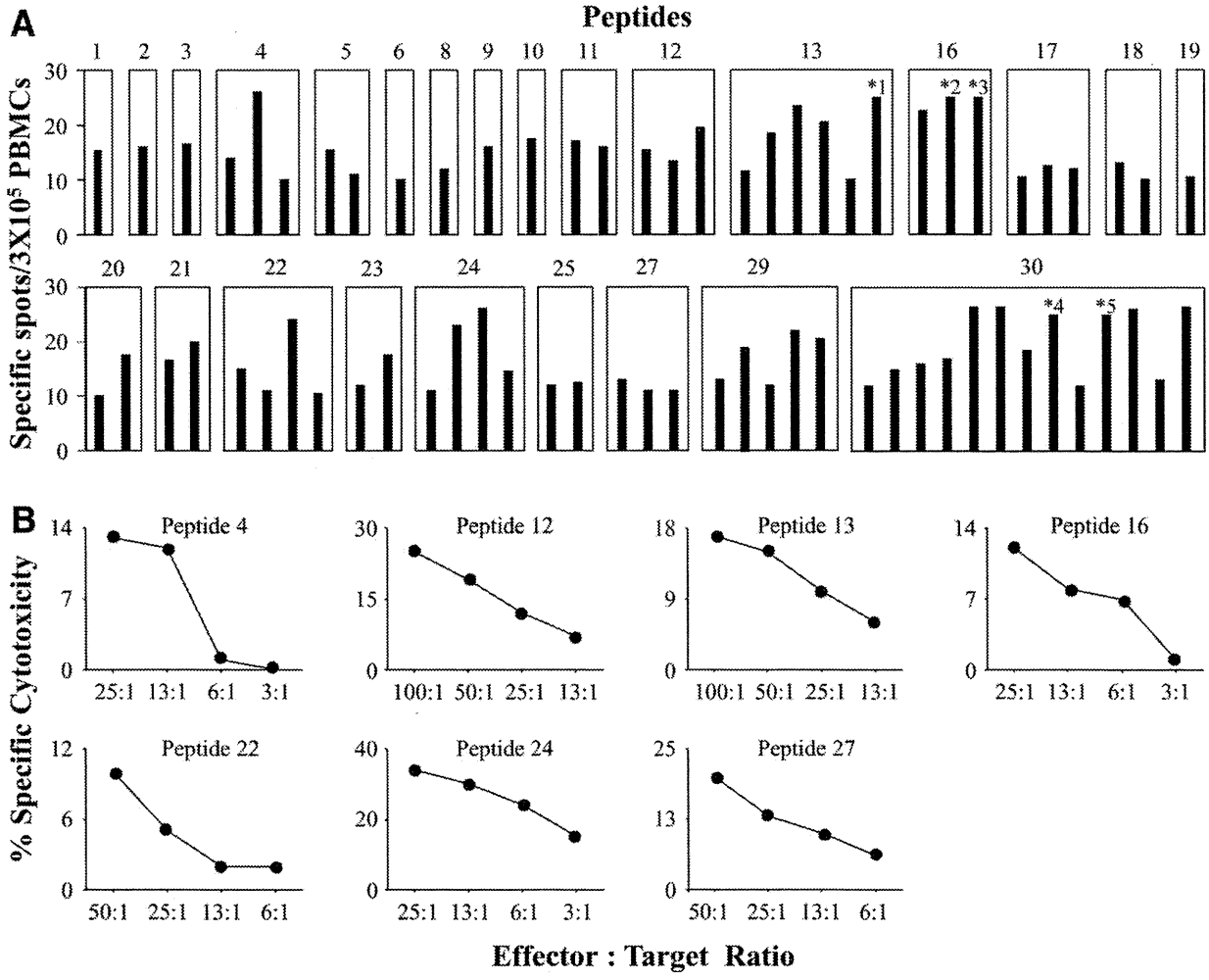

Fig. 2. Vigor of TAA-, HCV-, and CMV-derived peptide-specific T-cell responses. (A) The frequency of TAA-specific IFN-γ-producing T cells was analyzed by ELISPOT assay. Only positive responses are shown. Black bars indicate the response of one patient. *1, *2, *3, *4, and *5 denote 33, 60.5, 44, 92, and 67.5 specific spots, respectively. (B) Representative TAA-specific T-cell responses were also analyzed by CTL assay. T cell lines were generated from PBMC of the HLA-A24-positive HCC patients by stimulation with TAA-derived peptides (peptides 4, 12, 13, 16, 22, 24, and 27) (see Table 1). Expanded T cell lines were then tested for specific cytotoxicity against the corresponding peptides in a standard ^{51}Cr release assay at the indicated E:T ratios.

patients with chronic hepatitis C for 14 of 27 TAA-derived peptides (peptides 1, 2, 3, 4, 12, 16, 18, 19, 20, 21, 22, 24, 25 and 27) (Fig. 3C).

Enhancement of TAA-Specific T-Cell Responses After HCC Treatments. Several studies including our own have clarified that HCC treatments enhanced HCC-specific immune responses (19, 37, 38). In this study, we examined whether the enhancement was observed equally in all kinds of TAAs or specifically in some TAAs. For this purpose we measured the frequency of TAA-specific T cells before and after HCC treatment by ELISPOT assay in 12 cases who received transcatheter arterial embolization (TAE), radiofrequency ablation (RFA), or chemotherapy. The frequency of TAA-specific T cells increased in all patients and it was observed for 23 of 27 TAA-derived peptides (Fig. 4A). The enhancement was observed in the patients who received TAE, RFA, or chemotherapy and even in the patients without an increase in the frequency of CMV-specific T cells. Peptides 7, 14, 15, and 26, which were not recognized by T cells in all HCC patients before treatments (Fig. 1), were recognized by T cells in 1, 4, 1, and 5, respectively, of 12 patients after treatments. Representative results of enhancement of TAA-specific immune responses are shown in Fig. 4B. The frequency of TAA-specific T cells increased to 11-80 cells/300,000 PBMCs after treatments.

The enhancement of TAA-specific immune responses was also confirmed by cytokine secretion assay. Representative results are shown in Fig. 4C. In this patient (patient 25) the frequency of TAA-specific IFN-γ-producing $CD8^+$ T cells was increased from 0.4% to 1.4% of $CD8^+$ T cells after HCC treatment.

A

	TAA	HIV antigen	HCV antigen	CMV antigen
Normal donors	0/11(0%)	0/11(0%)	ND	4/11(36%)
Patients with HCV	10/29(34%)	0/29(0%)	8/29(28%)	15/29(52%)
Patients with HCC	24/31(77%)	0/31(0%)	5/31(16%)	13/31(42%)

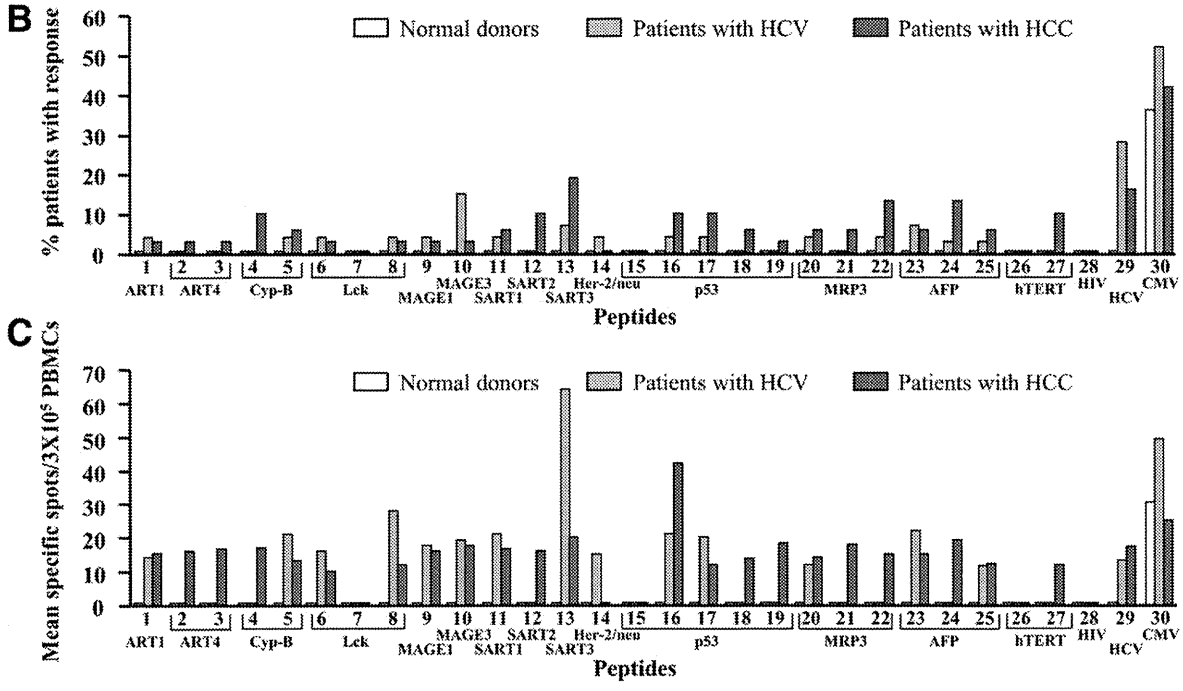

Fig. 3. Comparative analysis of TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses among three groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients. (A) Summary of the number of patients with a significant IFN-γ T-cell response to tumor-associated, HIV, HCV, and CMV antigens in each group. (B) Graph shows the percentage of patients in each group who showed a significant IFN-γ T-cell response to individual peptides. Peptide sequences are described in Table 1. (C) Mean frequency of peptide-specific IFN-γ-producing T cells in each group. The frequency of IFN-γ-producing T cells was analyzed by ELISPOT assay.

In this assay we also examined the naïve/effector/memory phenotype of these cells by the criterion of CD45RA/CCR7 expression.[39] Phenotypic analysis of TAA-specific, IFN-γ-producing memory $CD8^+$ T cells before and after treatment showed that the frequency of $CD45RA^-/CCR7^+$ central memory T cells was the highest, indicating that the posttherapeutic increase in these T cells is due to the increase in cells with this phenotype (Fig. 4D). In this patient the number of T cells with the $CD45RA^-/CCR7^+$ phenotype increased from 73 cells/300,000 PBMCs before treatment to 316 cells/300,000 PBMCs after treatment. Similar results were noted in five patients.

Blocking CTLA-4 Restores TAA-Specific T-Cell Responses. In previous studies including our own,[19,20,24] the CTL epitopes that correlate with the prevention of tumor progression or prognosis of HCC patients have not been identified. One of the reasons for this is considered to be that the naturally occurring T-cell responses to the epitopes are weak; therefore, recent tumor immunotherapeutic studies are moving toward modulation of T-cell responses.

CTLA-4 is recognized as a critical negative regulator of immune response; therefore, its blockade has been considered to contribute to antitumor activity.[27] In a recent study it was reported that blocking of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of CTLA-4 antibodies.[40] To examine whether similar occurs for immune response in HCC patients, we analyzed 32 separate TAA-specific T-cell responses in 15 HCC patients using 13 TAA-derived peptides. Incubation of T cells with CTLA-4 antibodies resulted in an increase of the number of TAA-specific T cells in 18 of 32 (56%) responses and in 9 of 15 (60%) patients (Fig. 5A). Fourteen and four patients showed increases of 1-10 and more than 10 TAA-specific T cells, respectively. Representative results of six patients are shown

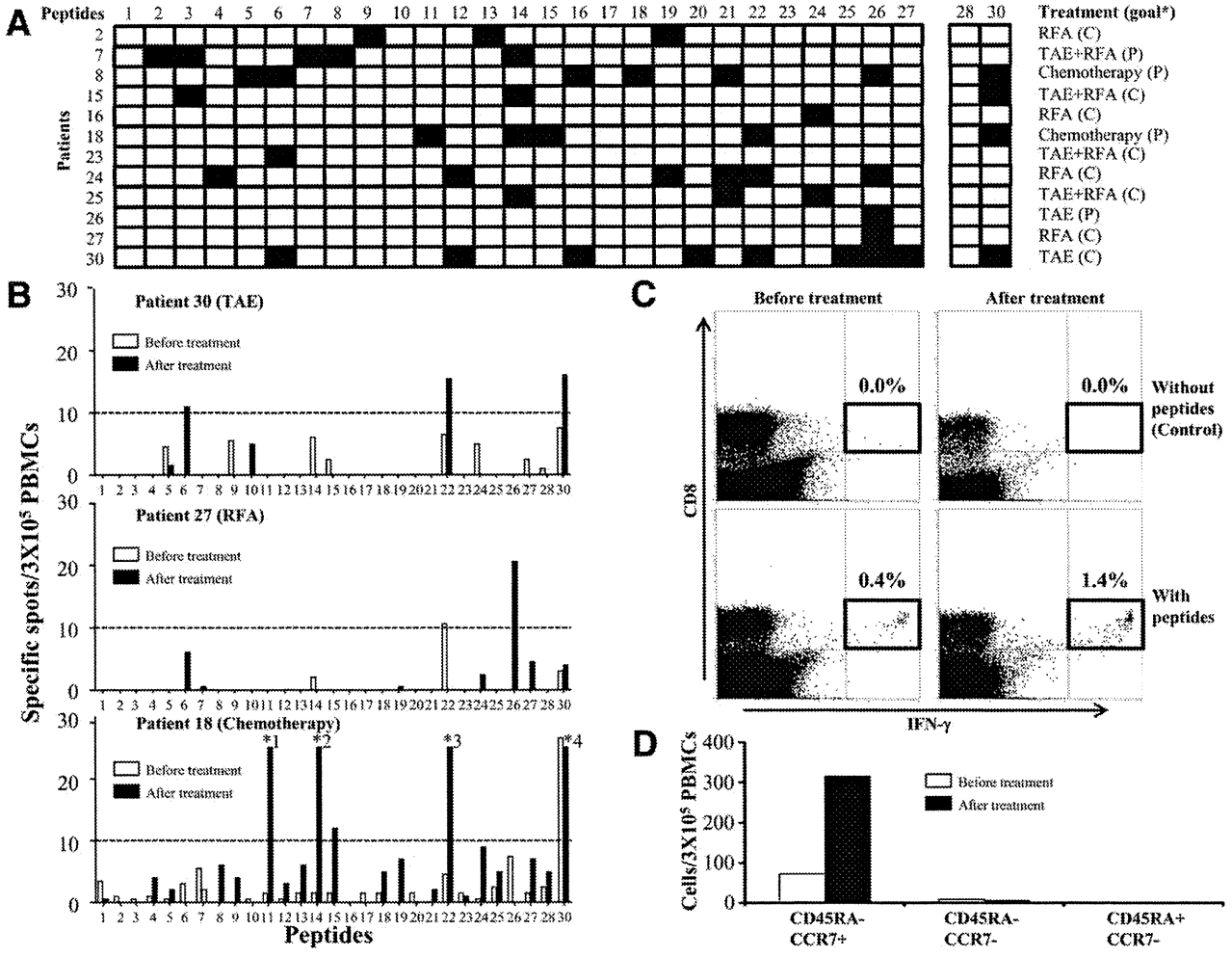

Fig. 4. Enhancement of TAA-specific T-cell responses in HCC patients after treatments. (A) Summary of patients and peptides with a significant increase of the number of IFN-γ-producing T cells (black boxes). A significant change in the IFN-γ response was defined as a more than 2-fold increase and the presence of more than 10 specific spots in ELISPOT assay after HCC treatments. The assays were performed in 12 HCC patients using 27 TAA-, HIV-, and CMV-derived peptides. Goal* shows the goal of HCC treatment. C and P denote "curative intention" and "palliative intention," respectively. (B) Representative results of ELISPOT assay are shown. White and black bars indicate the frequency of T cells before and after HCC treatments, respectively. *1, *2, *3, and *4 denote 53, 60, 80, and 121 specific spots, respectively. (C) Enhancement of TAA-specific T-cell responses was also analyzed by cytokine secretion assay. Representative results are shown (patient 25). PBMCs were pulsed with TAA-derived peptides (peptides 14, 21, and 24) for 16 hours and then analyzed for IFN-γ production. (D) IFN-γ-producing T cells were also examined for naïve/effector/memory phenotype by the criterion of CD45RA/CCR7 expression. The number of cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs. White and black bars indicate the frequency of TAA-specific IFN-γ-producing T cells before and after HCC treatments, respectively. The experiments were performed in five patients and similar results were observed.

in Fig. 5B. The magnitude of TAA-specific T-cell increase was statistically significant in four patients.

To examine the effect of CTLA-4 antibodies for production of other cytokines by T cells, we measured 27 kinds of human cytokines and chemokines in the medium of ELISPOT assay. Figure 5C shows the results of cytokine production in the well with positive T-cell responses against TAA-derived peptides. The various cytokines consisting of IL-1β, IL-4, IL-6, IL-10, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, MIP-1α, MIP-1β, RANTES, and TNF-α were increased in the medium with CTLA-4 antibodies compared with that without CTLA-4 antibodies. In contrast, increased production of these cytokines in the well without positive T-cell responses against TAA-derived peptides was not observed in medium either with or without CTLA-4 antibodies (Fig. 5D).

Discussion

In recent years, specific TAAs and their CTL epitopes have been identified in many tumors.[21] Several TAAs and their CTL epitopes, such as AFP, MAGE, and human telomerase reverse transcriptase (hTERT) have also been reported in HCC.[19,20,24,41] Although AFP-targeting immunotherapy could induce TAA-