Table 3. Univariate Analysis of Risks for Kidney Tubular Dysfunction in Patients With HIV Infection Treated With Tenofovir

Characteristic	OR	95% CI	*P* Value
Female sex	1.844	.204–16.67	.586
Age per 1 year	1.165	1.100–1.233	<.001
Weight per 1 kg decrement	1.076	1.021–1.135	.007
CD4 count per 1/μL decrement	1.002	.999–1.004	.261
Baseline eGFR per 1 mL/minutes/1.73 m^2 decrement	1.052	1.016–1.090	.004
Concurrent use of nephrotoxic drugs	1.559	.322–7.555	.581
Hepatitis B	0.721	.156–3.319	.674
C-reactive protein per 1 mg/dL	1.551	.689–3.494	.289
Hypertension	2.234	.843–5.922	.106
Dyslipidemia	0.578	.183–1.823	.349
Duration of treatment with tenofovir disoproxil fumarate (weeks)	0.999	.992–1.007	.888
ABCC2			
−24 CC	10.50	1.369–80.55	.024
1249 AA	7.828	1.609–38.10	.011
−24 CC plus 1249 AA	31.88	3.131–324.5	.003
2934 GG	1.358	.167–11.07	.775
ABCC4			
559 TT	4.912	.837–28.81	.078
912 TT	1.466	.531–4.042	.460
2269 AA	2.756	.530–14.34	.228
3348 GG	1.950	.510–7.463	.329
4135 GG	1.254	.450–3.494	.665
4976 CC	2.462	.925–6.547	.071
ABCC10			
526 GG	1.158	.360–3.725	.805
2759 TT	0.619	.220–1.738	.363
ABCB1			
2677 AA	7.828	1.609–38.10	.011

Abbreviations: CI, confidence interval; eGFR: estimated glomerular filtration rate; HIV, human immunodeficiency virus; OR, odds ratio.

[a] Due to low prevalence of minor alleles, rs56220353, rs11568630, and rs2274407 were not included in this analysis.

associated with tenofovir-induced KTD (OR, 2.497; 95% CI, .902–6.949; $P = .077$).

DISCUSSION

The present study demonstrated that genotype CC at position −24 and genotype AA at position 1249 of *ABCC2* gene are associated with HIV-1 infection. The effect of SNPs was more evident in patients with both −24 CC and 1249 AA homozygotes than in those with either homozygote only. The findings of this study resolve long-term controversy over the role of genetic

Table 4. Multivariate Analysis for the Risk of Tenofovir-Induced Kidney Tubular Dysfunction With Homozygotes at −24 and 1249 of *ABCC2* in Patients With HIV Infection

ABCC2	Adjusted OR	95% CI	*P* Value
Homozygote at −24 CC	20.08	1.711–235.7	.017
Homozygote at 1249 AA	16.21	1.630–161.1	.017
Homozygotes at −24 CC plus 1249 AA	38.44	2.051–720.4	.015

Each variable was adjusted for sex, age, weight, estimated glomerular filtration rate, and hypertension.

Abbreviations: CI, confidence interval; OR, odds ratio.

polymorphisms in tenofovir-induced KTD and confirm the effect of the SNPs in *ABCC2* gene in tenofovir-induced KTD.

CA haplotype (−24C, 1249A) of *ABCC2* was associated with tenofovir-induced KTD, whereas TG was a protective haplotype (Table 5). Izzedine et al [13] reported the role of CATC haplotype (−24C, 1249A, 3563T, 3972C) of *ABCC2* in KTD. However, 3563T did not play such role in this haplotype analysis, because the prevalence of 3563T is 0% in the Japanese, according to the HapMap data, and haplotype with only −24C plus 1249A still exhibited its effect on tenofovir-induced KTD (Table 5; www.hapmap.org). The reported association between tenofovir-induced KTD and 526G and 2759C of *ABCC10* described by Pushpakom et al [21] was also not reproduced in this study. Furthermore, SNPs in *ABCC4*, *SLC22A6*, and *ABCB1* investigated in the present study did not show a significant association with tenofovir-induced KTD (Table 3).

Three main aspects of our study are important. First, this is the first study to our knowledge that elucidated the effect of SNPs on tenofovir-induced KTD conducted in a country other than European countries or the United States. Our study examined Japanese patients of genetic background different from patients of previous studies, which consisted mostly of whites. While SNPs −24C and 1249A of *ABCC2* have been speculated to correlate with tenofovir-induced KTD in previous studies, the present study confirmed that these SNPs are risk factors for tenofovir-induced KTD in nonwhites.

The result that the SNPs in *ABCC2* are a risk for tenofovir-induced KTD can also be applied to patients with other genetic backgrounds who host SNPs −24C and 1249A. Notably, the impact of SNPs on tenofovir-induced KTD might be more significant in Africans and Indians than in Japanese or whites, considering that the allele frequencies of −24C and 1249A are higher in these population according to the HapMap data (−24C; Africans 96.9%, Indians 92.6%, Japanese 80.8%, whites 81.9%, 1249A; Africans 21.7%, Indians 30.7%, Japanese 8.9%, whites 23.7%; www.hapmap.org).

Second, the study was designed to evaluate the exclusive effect of SNPs on tenofovir-induced KTD by excluding

Table 5. Association Between Haplotype in *ABCC2* and *ABCC4* and Kidney Tubular Dysfunction

SNP Marker/Haplotype	Allele	Allele/Haplotype Frequency, %		OR (95% CI)[a]	*P* Value
		KTD Group (n = 19)	Control Group (n = 171)		
ABCC2					
−24 C → T	C	97.4	78.4	10.22 (1.658–419.8)	.003
1249 G → A	A	28.9	12.3	2.91 (1.345–6.296)	.011
ABCC2 haplotype	CA	28.9	12.3	2.91 (1.295–6.221)	.011
	TG	2.6	21.6	0.098 (.002–.603)	.003
ABCC4					
559 G → T	T	21.1	12.3	1.905 (.705–4.614)	.213
4976 T → C	T	48	55.3	0.746 (.375–1.470)	.399
ABCC4 haplotype					
TT	TT	17.6	7.9	2.497 (.902–6.949)	.077

Abbreviations: CI, confidence interval; KTD, kidney tubular dysfunction; OR, odds ratio; SNP, single-nucleotide polymorphism.

[a] ORs and *P* values are for comparisons of allele/haplotype frequencies between the kidney tubular dysfunction and control groups.

possible predisposing factors for KTD, for example, active infection, malignancies, diabetes mellitus, and preexisting renal impairment, which are known risks for KTD [35]. Patients who showed no HIV-1 viral suppression were also excluded. Furthermore, the enrolled patients were Japanese only, and this helped to examine a study population with comparatively similar genetic background. The study population was also on the same antiretroviral regimen (ritonavir-boosted darunavir plus tenofovir/emtricitabine), and this also helped to evaluate more precisely the effect of SNPs, because plasma concentration of tenofovir is affected by concomitant antiretrovirals and the delta change in plasma tenofovir concentration likely differs in the presence of each concomitant drug [26].

Third, SNPs were examined in 190 patients in this study. To our knowledge, the number of enrolled patients is the largest among the studies that have so far examined the effect of SNPs on tenofovir-induced KTD. Thus, this feature provided the study a higher statistical power than previous studies.

Why are polymorphisms in *ABCC2* a risk for tenofovir-induced KTD, even though it is controversial whether MRP2 plays a role in the excretion of tenofovir via the luminal membrane? [18, 20] The exact mechanism has not been determined yet, but we speculate 2 hypotheses. First, there might be unknown endogenous substances that influence tenofovir nephrotoxicity in renal tubular cells, and SNPs in *ABCC2* modulate the function or transportation of such substances [15]. Second, MRP2 may indeed take part in transporting tenofovir, because various substances including methotrexate are reported to be a substrate of MRP2, and *ABCC2* mutation alters excretion of those substances [36, 37]. Further studies are warranted to elucidate the exact mechanism of these SNPs on tenofovir-induced KTD. Furthermore, the impact of these SNPs on KTD with long-term TDF use needs to be evaluated in prospective studies.

Several limitations need to be acknowledged. First, not all polymorphisms in genes of the targeted transporter proteins were examined. Thus, we might have missed other important SNPs on the function of tenofovir transportation. There might be other unknown transporter proteins for tenofovir excretion in the kidney that contribute to susceptibility to tenofovir-induced KTD as well. Second, the diagnostic criteria for TDF-induced KTD are not uniformly established in the field and are different in the published studies. The criteria applied in this study are not entirely similar to the ones used in previous studies that examined the role of SNPs in tenofovir-induced KTD. However, by excluding other predisposing factors for KTD and enrolling a large number of patients, this study succeeded in providing a clear-cut association between SNPs and tenofovir-induced KTD.

In conclusion, the present study demonstrated that SNPs in *ABCC2* associate with tenofovir-induced KTD in Japanese patients, in a setting that excluded other predisposing factors. Assessment of renal tubular function is more cumbersome and costly to monitor than serum creatinine. However, monitoring tubular function is clinically important, because undetected long-term tubular dysfunction might lead to premature osteopenia due to phosphate wasting and accelerated progression of renal dysfunction. Close monitoring of tubular function is warranted in patients with *ABCC2* −24C and 1249A under TDF treatment.

Notes

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Differential Clade-Specific HLA-B*3501 Association with HIV-1 Disease Outcome Is Linked to Immunogenicity of a Single Gag Epitope

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The strongest genetic influence on immune control in HIV-1 infection is the HLA class I genotype. Rapid disease progression in B-clade infection has been linked to HLA-B*35 expression, in particular to the less common HLA-B*3502 and HLA-B*3503 subtypes but also to the most prevalent subtype, HLA-B*3501. In these studies we first demonstrated that whereas HLA-B*3501 is associated with a high viral set point in two further B-clade-infected cohorts, in Japan and Mexico, this association does not hold in two large C-clade-infected African cohorts. We tested the hypothesis that clade-specific differences in HLA associations with disease outcomes may be related to distinct targeting of critical CD8$^+$ T-cell epitopes. We observed that only one epitope was significantly targeted differentially, namely, the Gag-specific epitope NPPIPVGDIY (NY10, Gag positions 253 to 262) ($P = 2 \times 10^{-5}$). In common with two other HLA-B*3501-restricted epitopes, in Gag and Nef, that were not targeted differentially, a response toward NY10 was associated with a significantly lower viral set point. Nonimmunogenicity of NY10 in B-clade-infected subjects derives from the Gag-D260E polymorphism present in ~90% of B-clade sequences, which critically reduces recognition of the Gag NY10 epitope. These data suggest that in spite of any inherent HLA-linked T-cell receptor repertoire differences that may exist, maximizing the breadth of the Gag-specific CD8$^+$ T-cell response, by the addition of even a single epitope, may be of overriding importance in achieving immune control of HIV infection. This distinction is of direct relevance to development of vaccines designed to optimize the anti-HIV CD8$^+$ T-cell response in all individuals, irrespective of HLA type.

Several genome-wide association studies now indicate that the host HLA class I genotype is the major genetic determinant of HIV-1 disease progression (19, 20, 61). Previously it had been established that differences in HLA allele expression have a substantial impact on HIV disease outcome, in both B-clade (10, 19, 20, 59) and C-clade (38, 44, 54, 63) infection. Variation at the HLA-B locus has the greatest impact on viral set point (20, 38). This may result from the increased diversity of HLA-B compared to non-HLA-B alleles (28), affecting the repertoire, protein specificity, and peptide-binding characteristics of epitopes presented by HLA-B alleles (38, 39, 41). In addition, HLA-Bw4 alleles can act as KIR ligands and modulate an NK response, with certain HLA-KIR combinations resulting in selection pressure on HIV and/or significantly influencing viral set point (2, 3, 52, 68).

The mechanisms by which certain HLA alleles are consistently linked with particular HIV disease outcomes remain unresolved. Several possible mechanisms have been proposed. First, HLA-associated immune control has been linked to the specificity of the CD8$^+$ T-cell response (39, 54). In this way, HLA alleles such as HLA-B*57 or HLA-B*27, associated with immune control (4, 46, 59), restrict dominant Gag-specific responses, escape from which results in a substantial reduction in viral replicative capacity (13, 15, 46, 53, 65). In contrast, HLA alleles such as HLA-B*35, associated with rapid disease progression (12), restrict dominant epitopes in Nef, Env, and other non-Gag proteins (7, 39, 58, 67, 69, 72, 73).

A second mechanism proposed for the association of particular HLA types with characteristic HIV disease outcomes is through an impact on antiviral NK activity, since certain HLA alleles have the potential to act as KIR ligands. The HLA alleles associated with lowest viral set point tend to be HLA-Bw4 alleles (22). HLA-Bw4 alleles expressing Ile at HLA residue 80 significantly reduce the viral set point in combination with either KIR3DS1 or KIR3DL1 (51, 52). However, the impact of HLA-KIR combinations only partially explains the effect of protective alleles such as HLA-B*27 and HLA-B*57 or of disease susceptibility alleles such as HLA-B*35 (5, 52).

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A third mechanism, more recently proposed (41), suggests that disease susceptibility (61) HLA alleles such as HLA-B*0702 and HLA-B*3501 have peptide-binding motifs such that large numbers of self peptides can bind, and hence a relatively large proportion of the T-cell receptor (TcR) repertoire would be lost through negative selection of autoreactive T cells in the thymus. In contrast, protective alleles such as HLA-B*2705 and HLA-B*5701 have more restrictive peptide-binding motifs, with a requirement for Arg at P2 in the case of HLA-B*2705 and a strong preference for Trp at the C-terminal position in the case of HLA-B*5701 (50). This would result in fewer autoreactive T cells being deleted in the thymus via negative selection and therefore a relatively large TcR repertoire remaining to accommodate the challenge of epitope variation inevitably presented by viruses such as HIV.

An additional mechanism proposed to explain the status of HLA-B*3503 as linked with more rapid disease progression than HLA-B*3501 (25), from which it differs by only one amino acid, derives from the observation that HLA-B*3503 binds with significantly greater affinity than HLA-B*3501 to immunoglobulin-like transcript 4 (ILT-4), an inhibitory major histocompatibility complex (MHC) class I receptor expressed on dendritic cell (34). These data suggest the possibility that dendritic cell function may be significantly affected by a variety of HLA molecules, thereby explaining a range of differential HLA associations with HIV disease outcome.

We here describe an observation that allows us to test the first of these hypothetical mechanisms. While HLA-B*3501 is associated with less rapid progression to HIV disease than the less common subtypes of HLA-B*35 in Caucasians, B*3502 and B*3503 (25), HLA-B*3501 itself has also been associated with higher-than-average viremia in B-clade HIV-1 infection (42). For example, in a recent study of 3,622 B-clade-infected study subjects, HLA-B*3501 was strongly associated with HIV disease progression (61). However, in a cohort of C-clade-infected study subjects (n = 1,210) in Durban, South Africa, we noted that HLA-B*3501 is somewhat protective: viral set points tend to be somewhat lower in HLA-B*3501-positive subjects. Indeed, having removed the effect of HLA-B*57, HLA-B*5801, HLA-B*1801, and HLA-B*5802, the alleles having the strongest impact on viral set point and absolute CD4 count (38), HLA-B*3501 was the HLA-B allele associated with the highest absolute CD4 counts (44, 54) in this C-clade-infected cohort.

We show here, first, that this observation of clade specificity of the HLA-B*3501 effect on viral set point could be replicated in two additional B-clade-infected cohorts, namely, in Japan and in Mexico, and in an additional C-clade-infected cohort in Botswana. We then tested the hypothesis that the clade-specific difference in HLA-B*3501-associated HIV disease outcome could be related to altered specificity of the CD8$^+$ T-cell response. Based on the "Gag hypothesis" as described above, HLA-B*3501-restricted responses in C-clade infection would tend to be more Gag directed and less Nef/Env directed than in B-clade infection.

MATERIALS AND METHODS

Ethics statement. Ethics approval was given by the following: the University of KwaZulu-Natal Review Board and the Massachusetts General Hospital Review Board (Durban cohort); the Office of Human Research Administration, Harvard School of Public Health, and the Health Research Development Committee, Botswana Ministry of Health (Gaborone cohort); the Oxford Research Ethics Committee (Thames Valley and other cohorts); and the Ethics Committees of Kumamoto University and National Centre for Global Health and Medicine (Kumamoto cohort). Study subjects from all cohorts gave written informed consent for their participation.

Study cohorts. We studied a total of 3,132 adults with chronic, antiretroviral therapy (ART)-naïve HIV-1 infection, recruited from six cohorts as follows: (i) Durban, South Africa (C clade; n = 1,218), as previously described (38, 39, 46, 54); (ii) Gaborone, Botswana (C clade; n = 514) via the Mma Bana study, as previously described (66); (iii) Kumamoto, Japan (B clade; n = 242), as previously described (37); and (iv) Mexico City, Mexico (B clade; n = 771), as previously described (6) (see Table S1 in the supplemental material); (v) the Thames Valley cohort, United Kingdom (mixed clades; n = 237), as previously described (60, 62); and (vi) a B-clade-infected cohort of 150 subjects drawn from multiple ethnicities, also as previously described (24). Viral loads were determined using Roche Amplicor version 1.5 assay; CD4$^+$ T-cell counts were determined by flow cytometry.

HLA typing and classification. HLA typing from genomic DNA was undertaken by sequence-based typing as previously described (38). Locus-specific PCR products of exons 2 and 3 were amplified and sequenced. In the Kumamoto cohort, 32/37 subjects with HLA-B*35 were typed to 4 digits, and all 32 of these were HLA-B*3501 positive; because of this, and because of a previous analysis of 1,018 Japanese subjects (36) which showed that 158/159 subjects with HLA-B*35 had HLA-B*3501, the remaining 5 Japanese subjects were designated HLA-B*3501 positive. Likewise, in the southern African cohorts, 96/102 HLA-B*35-positive subjects typed to 4 digits were HLA-B*3501 positive. For 23 Durban subjects in whom HLA-B*35 typing had been undertaken only to 2-digit resolution, we used an HLA completion tool (http://atom.research.microsoft.com/HLACompletion) (47) to predict the most likely 4-digit HLA-B*35 allele. In all cases HLA-B*3501 was predicted as the 4-digit type with a high level of statistical certainty (probability of B*3501, 0.86 to 0.98; median, 0.97). For this reason, we designated all 23 Durban subjects with HLA-B*35 typed to 2-digit resolution as HLA-B*3501.

Definition of HLA-B*3501-restricted epitopes. To define a comprehensive list of HLA-B*3501-restricted epitopes, we identified previously characterized epitopes from studies of predominantly B-clade-infected subjects (Los Alamos "A list"; www.lanl.gov) (48) and also identified five novel HLA-B*3501-restricted epitopes by testing recognition of 410 overlapping 18-mer peptides in a cohort of C-clade-infected subjects (see Table S2 in the supplemental material). One of these (HA9) has, since the start of this study, now been confirmed by another group (74). From this dual approach, 13 HLA-B*3501-restricted epitopes were identified for further analysis (Table 1).

IFN-γ ELISpot assays. Gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assays were undertaken using fresh or cryopreserved peripheral blood mononuclear cells (PBMCs). We screened for HIV-1-specific responses statistically associated ($q < 0.05$) with the expression of HLA-B*3501 by testing a total of 1,010 chronically infected subjects (n = 795 from Durban; n = 215 from the Thames Valley) against a panel of 410 overlapping peptides (OLPs) spanning the entire HIV proteome, as previously described (38, 39, 54). Significant associations were determined using Fisher's exact test and corrected for multiple comparisons using a q value (false-detection rate [FDR]) approach as previously described (11, 40, 54).

In order to screen subjects with HLA-B*3501 for specific responses to HLA-B*3501 epitopes, B-clade-infected subjects were tested for IFN-γ responses to optimal peptides (Japan, n = 30) or against overlapping

TABLE 1 Thirteen HLA-B*3501-restricted epitopes in HIV-1 from Gag, Pol, Rev, Env, and Nef proteins[a]

Protein	Clade	Epitope sequence[b] P2 ↓	C terminus ↓	HXB2 position	Epitope designation
p24 Gag	B	HPVHAGPI	A	Gag 216-224	Gag HA9*
	C	--------	-		
	B	NPPIPVGEI	Y	Gag 253-262	Gag NY10
	C	-------D-	-		
RT	B	TVLDVGDA	Y	Pol 262-270	RT TY9
	C	--------	-		
	B	VPLDKDFRK	Y	Pol 273-282	RT VY10
	C	----E---	-		
	B	NPDIVIYQ	Y	Pol 330-338	RT NY9
	C	--E-----	-		
	B	EPIVGAETF	Y	Pol 587-596	RT EY10*
	C	---A-----	-		
Int	B	IPAETGQETA	Y	Pol 804-814	Int IY11*
	C	----------	-		
Rev	B	KTVRLIKFL	Y	Rev 14-23	Rev KY10, Rev QY10*
	C	QA--I--I-	-		
gp120	B	VPVWKEATTT	L	Env 42-52	Env VL11
	C	-----K--	-		
	B	DPNPQEVV	L	Env 78-86	Env DL9
	C	------M-	-		
gp41	B	TAVPWNAS	W	Env 606-614	Env TW9
	C	------S-	-		
Nef	B	VPLRPMT	Y	Nef 73-81	Nef VY8, Nef VF8
	C	-------	F		
	B	YPLTFGWC	Y	Nef 135-143	Nef YY9, Nef YF9*
	C	--------	F		

[a] The 13 epitopes include 8 from the Los Alamos database "A list" (www.lanl.gov) and 5 new HLA-B*3501-restricted optimal epitopes (indicated by asterisks).
[b] The B- and C-clade consensus sequences of each epitope are listed; a dash indicates no difference between clades. Residues at position 2 and at the C terminus are in bold.

peptides in a previously described B-clade cohort (23, 24) (n = 44). C-clade-infected subjects (n = 42) were tested for responses to the C-clade version of the same epitopes using the respective 18-mer peptides containing the HLA-B*3501 epitopes.

Viruses from all study subjects in the Japan cohort were sequenced to confirm clade of infection, and only those subjects who were B-clade infected were included in the study (one subject who was A-clade infected was excluded). Likewise C-clade infection was confirmed in >99% of the southern African study subjects. The B-clade-infected subjects were tested for recognition of the version of the peptides corresponding to the B-clade consensus sequence in Japan, and the C-clade-infected subjects were tested for recognition of the version corresponding to the C-clade consensus sequence (the 2006 Durban and other Southern African consensus sequence). Using previously established criteria (38, 39), a response of 100 spot-forming cells (SFC)/10^6 PBMC was defined as significantly above the background response in control wells.

Epitope fine mapping and HLA class I tetramer assay. We confirmed HA9 (HPVHAGPIA; Gag positions 216 to 224) as an HLA-B*3501-restricted optimal epitope via assays of PBMCs in subject R051 (HLA-A*0101, -A*3002, -B*1801, -B*3501, -Cw*0401, -Cw*0501) against the optimal peptide and four truncations (±1 amino acid at the C and N termini); this experiment was performed in triplicate. Likewise, NY10 (NPPIPVGDIY; Gag positions 253 to 262) was optimized against the PY9 (PPIPVGDIY; Gag positions 254 to 262) using responder PBMCs from subject H033 (HLA-A*3601, -A*7401, -B*3501, -B*5301, -Cw*0401, -Cw*0401) in an IFN-γ ELISpot peptide titration assay.

The corresponding peptide responses were validated using HLA class I tetramers and controlled by a mismatched HLA-B*4201 tetramer. A pretitrated concentration of phycoerythrin (PE)-conjugated tetramers (43) was used to stain PBMCs, which were incubated for 30 min and stained with pretitrated extracellular antibodies CD8-Pacific Blue (BD Pharmingen) and CD3-Pacific Orange (Invitrogen). Dead cells were excluded using the Vivid LIVE/DEAD marker (Invitrogen). For NY10-Gag dual-tetramer staining, PBMCs from subject OX030 were stained *ex vivo* or *in vitro* expanded for 12 days using 10 μg/ml of NY10-260D or NY10-260E in culture medium RPMI 1640 (Gibco) supplemented with 10% human serum, 1% penicillin-streptomycin (Invitrogen), and 10% T-cell growth factor (Helvetica), costained with HLA-B*3501-NPPIPVGDIY (PE conjugated) and HLA-B*3501-NPPIPVGEIY (allophycocyanin [APC] conjugated) pretitrated tetramers (*ex vivo* PBMCs) or in 2-fold titrations (cytotoxic T lymphocytes [CTLs]), and subsequently stained with extracellular antibodies as described above.

Intracellular cytokine staining. PBMCs from subject KI-705 were stimulated with NY10-260D (NPPIPVGDIY) or NY10-260E (NPPIPGVEIY) (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human interleukin-2 [IL-2]). After 14 days in culture, the cells were assessed for IFN-γ production. Briefly, bulk cultures were cocultured with C1R cells expressing HLA-B*3501 pulsed with NY10-260E or NY10-260D peptide for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the cocultures were continued for additional 4 h. Cells were stained with phycoerythrin (PE)-labeled anti-CD8 monoclonal antibody (Dako Corporation, Glostrup, Denmark) and subsequently fixed (4% paraformaldehyde), permeabilized (0.1% saponin and 20% NCS in phosphate-buffered saline), and intracellularly stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ monoclonal antibody (MAb) (PharMingen, San Diego, CA). Samples were acquired on a FACSCalibur instrument within 24 h of staining and data analyzed using FlowJo version 8.8.6.

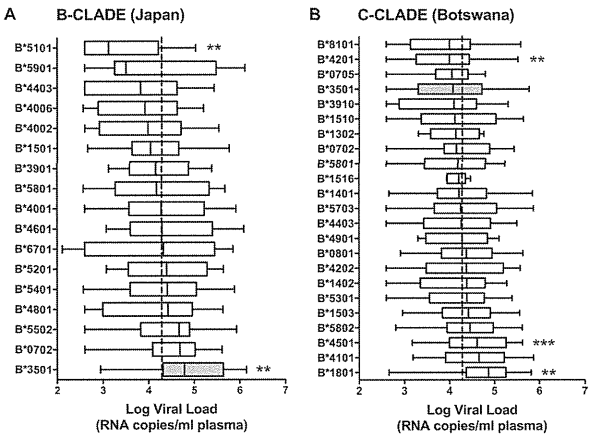

FIG 1 Ranking of HLA-B alleles with respect to median viral load (VL) in chronic HIV-1 infection in B- and C-clade-infected study cohorts. Boxes show median and 25th and 75th centiles; whiskers show 10 to 90% confidence intervals. HLA-B*3501 is highlighted in gray. Dashed lines indicate median VL for the whole cohort. P values by Mann-Whitney test, comparing VL for subjects with each allele to the whole population: ***, $P < 0.0001$; **, $P < 0.001$. Alleles represented are those occurring at ≥0.5% phenotypic frequency and for which a minimum of 5 subjects had VL data available. (A) Kumamoto, Japan (median VL, 19,500 RNA copies/ml). (B) Gaborone, Botswana (median VL, 19,150 RNA copies/ml). For equivalent data for Durban, South Africa, see reference 55.

Generation of mutant virus NL43-2 E260D and epitope processing assay. The NL43-2-Gag260D mutant virus was generated by introducing the Gag260D mutation into the NL43-2 Gag260E backbone using site-directed mutagenesis (Invitrogen). After virus generation, 721.221-CD4-B*3501 and 721.221 target cells were infected with NL43-2 B-clade WT(Gag260E) or NL4-32-Gag260D mutant virus. The infection rates were determined by the level of intracellular p24-positive cells stained with FITC-conjugated anti-p24 MAb (KC57-FITC; BD Biosciences) and followed over 6 days. When the level of p24-positive target cells reached 80%, the Gag NY10-specific $CD8^+$ T-cell line and the control Pol-EY10 $CD8^+$ T-cell clone was cocultured with the target cells for 5 h in the presence of brefeldin A and subsequently stained for intracellular IFN-γ as described above. The level of IFN-γ-positive $CD8^+$ T cells after coculture was used as a measure of the level of specific epitope presentation and controlled by uninfected HLA-matched and infected HLA-negative 721.221 cells. Peptide-pulsed HLA-matched target cells were used as positive controls for optimal epitope presentation. Samples were acquired on a FACSCalibur instrument within 24 h of staining and data analyzed using FlowJo version 8.8.6

Peptide-MHC binding studies. HLA-peptide binding studies were undertaken using a luminescent oxygen channeling immunoassay (LOCI) as previously described (29). We tested binding for 12 HLA-B*3501 epitopes as shown in Table S3 in the supplemental material. Binding assays were performed in quadruplicate; the reported result is the mean of the four values obtained.

Stability of binding (binding half-life) was determined as described previously (30). Briefly, biotinylated HLA-I heavy chain, ^{125}I-labeled beta-2-microglobulin (B2m), and peptide were allowed to fold into peptide–HLA-I complexes in streptavidin-coated scintillation microplates (Flashplate Plus; Perkin-Elmer, Boston, MA) for 24 h at 18°C. Excess unlabeled B2m was added, and dissociation was initiated by placing the microplate in a scintillation reader (TopCount NXT; Perkin-Elmer, Boston, MA) operating at 37°C. The scintillation signal was monitored by continuous reading of the microplate for 24 h. Half-lives were calculated from dissociation curves using the exponential decay equation in Prism v.5.0a (GraphPad, San Diego, CA). Assays were performed in duplicate; the mean value from two experiments is reported.

Statistical analysis. Statistical analysis was undertaken using GraphPad Prism v.5.0a (GraphPad, San Diego, CA). To define the sites of new putative HLA-B*3501 epitopes, relationships between HIV-1 sequence polymorphisms and HLA class I expression and between ELISpot responses and HLA class I expression were determined using Fisher's exact test (corrected for viral lineage in the case of sequence analysis) and corrected for multiple comparisons using a q value (false-detection rate), as previously described (11, 54).

RESULTS

Consistent differential HLA-B*3501-association with viral set point in B- and C-clade infection. We first sought to test the consistency of our initial observation that, in contrast to its impact in B-clade infection (7, 21, 59), HLA-B*3501 is not associated with high viral set point in C-clade infection (38, 44). In B-clade-infected cohorts in Mexico and in Japan, HLA-B*3501 is associated with a high viral set point (P = 0.06 and P = 0.0005, respectively) (Fig. 1 and 2). In contrast, in a C-clade-infected Botswanan cohort, HLA-B*3501 is somewhat protective, although this did not reach statistical significance (Fig. 1 and 2).

HLA-B*3501 is also associated with higher absolute $CD4^+$ T-cell counts in subjects with C-clade infection (Durban, P = 0.06;

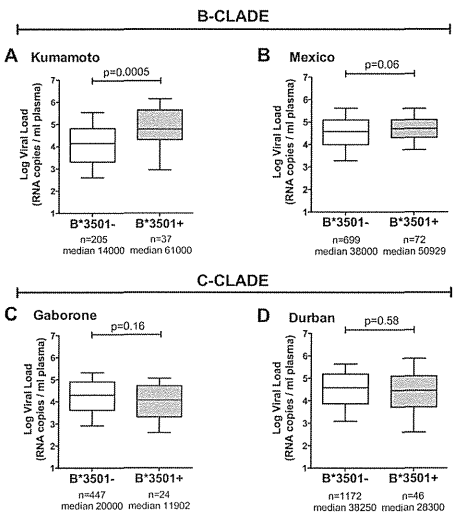

FIG 2 Median viral load in subjects with and without HLA-B*3501 in B- and C-clade-infected study cohorts. Boxes show median and 25th and 75th centiles; whiskers show 10 to 90% confidence intervals. (A) Kumamoto, Japan (B clade); (B) Mexico City, Mexico (B clade); (C) Gaborone, Botswana (C clade); (D) Durban, South Africa (C clade). *P* values are by the Mann-Whitney test.

Gaborone, $P = 0.16$; $P = 0.01$ when data were pooled; median absolute CD4 counts, 439 versus 369 cells/mm^3 in HLA-B*3501-positive versus HLA-B*3501-negative subjects) (data not shown). In contrast, HLA-B*3501 is associated with lower absolute CD4 counts in subjects with B-clade infection (Mexico, $P = 0.01$; Japan, $P = 0.3$; $P = 0.01$ when data were pooled; median absolute CD4 counts, 249 versus 370 cells/mm^3 in HLA-B*3501-positive versus HLA-B*3501-negative subjects) (data not shown).

Thus, in two large C-clade-infected cohorts, HLA-B*3501 is associated with lower viral loads and higher CD4$^+$ T-cell counts in chronic HIV infection, whereas in B-clade-infected cohorts, such as those studied in Japan and in Mexico, HLA-B*3501 tends to be associated with a higher viral set point and lower absolute CD4 count.

HLA-B*3501-restricted CD8$^+$ T-cell responses in B- and C-clade infection. In order to investigate whether the observed difference in HLA-B*3501-associated HIV disease outcome in B- and C-clade-infected cohorts is related to clade-specific differences in the CD8$^+$ T-cell activity, we measured responses in HLA-B*3501-positive subjects infected with B- or C-clade virus to a comprehensive panel of HLA-B*3501-restricted epitopes (Table 1). This panel comprised epitopes previously defined from studies of B-clade-infected subjects with HLA-B*3501 and published in the Los Alamos Immunology database "A list" (www.hiv.lanl.gov) (48), together with 5 additional novel epitopes that were identified by analysis of CD8$^+$ T-cell responses in a cohort of 1,010 study subjects (40) to a panel of 410 overlapping 18-mer peptides (OLPs) spanning the C-clade proteome (see Table S2 in the supplemental material). An illustration of the approach that was used to identify these HLA-B*3501-restricted epitopes is shown for HPVHAGPIA (Gag positions 216 to 224) (HA9) (see Fig. S1 in the supplemental material), which was recently also described by another group (74) as a p24 Gag epitope restricted by HLA-B*3501.

For all the epitopes identified that were not listed in the Los Alamos Immunology database (www.hiv.lanl.gov) (48), in each case strong binding avidities to HLA-B*3501 (with the K_d [dissociation constant] ranging between 1 and 55 nM) were demonstrated (data not shown), and a CD8$^+$ T-cell response to each was detected in ≥2 study subjects tested (see below). In the process of validating the novel and previously published HLA-B*3501-restricted epitopes using HLA-class I tetramers (40), we noted one epitope that had been previously identified via an epitope prediction approach as PPIPVGDIY (PY9) (Gag positions 254 to 262) (64). We demonstrated that the true optimal epitope is the 10-mer NPPIPVGDIY (NY10) (Gag positions 253 to 262), which is consistently recognized at <1/1,000 of the concentration of PY9 (Fig. 3A). HLA-B*3501 tetramer staining of antigen-specific cells was readily observed using the 10-mer NY10 (Fig. 3B) but was never achieved using the 9-mer PY9. This process of distinguishing the correct epitope, NY10, from the incorrect epitope, PY9, was of crucial significance in understanding the differential impact of HLA-B*3501 in B- and C-clade HIV infection (see below).

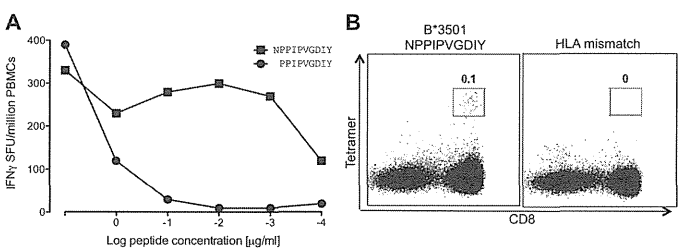

FIG 3 Optimization of the NY10 Gag epitope (NPPIPVGDIY). (A) IFN-γ ELISpot responses to titrated amounts of the 9-mer PPIPVGDIY versus the 10-mer NPPIPVGDIY peptides made by an HLA-B*3501-positive adult subject with chronic B-clade HIV-1 infection (Thames Valley subject H033, HLA-A*3601, -A*7401, -B*3501, -B*5301, -Cw*0401, -Cw*0401). (B) Unequivocal definition of the correct HLA-B*3501-restricted optimal epitope NY10 using an HLA-B*3501–NY10 tetramer to stain the NY10 responder PBMCs from the same subject (H033) as used for panel A. Results from one representative of two independent experiments are shown.

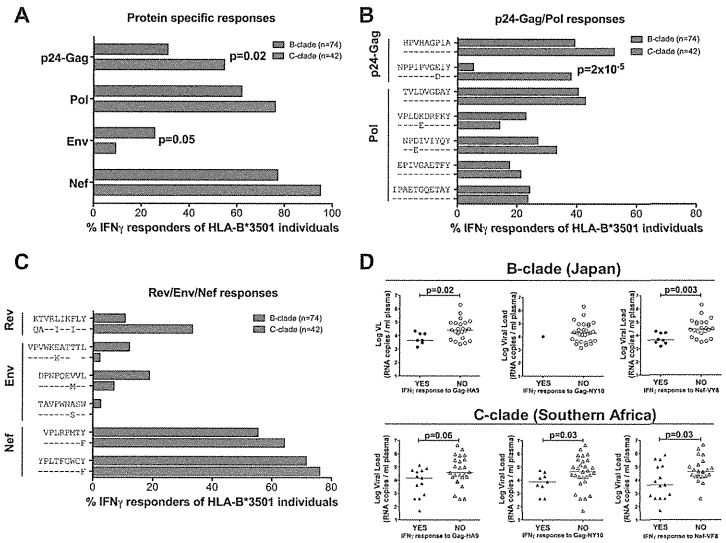

FIG 4 Percentage of HLA-B*3501-positive subjects making IFN-γ ELISpot responses to 13 HLA-B*3501-restricted epitopes in B- and C-clade infection and the impact on viral load to HA9 Gag, NY10 Gag and VY/VF8 Nef responses controlled by HLA-B*3501 matched nonresponding individuals. (A) Responses expressed as protein specific were obtained by pooling the percentage of adult HLA-B*3501-positive subjects with B-clade infection (Kumamoto, Japan) making IFN-γ ELISpot responses to individual HLA-B*3501-restricted optimal peptides ($n = 30$ subjects) pooled with another B-clade cohort (23, 44) screened against 18-mer overlapping peptides containing the optimal epitopes ($n = 44$ subjects) (blue) (total of 74 B-clade-infected subjects) and compared to adult subjects with C-clade infection (southern African subjects) tested against C-clade consensus overlapping peptides containing the corresponding optimal peptides ($n = 42$ subjects) (red). (B) Responses as in panel A but shown for individual epitopes within Gag and Pol proteins. (C) Responses as in panel A but shown for individual epitopes within Rev, Env, and Nef proteins. (D) Comparison of viral load between responders and nonresponders for B-clade-infected Japanese subjects ($n = 30$), based on responses to optimal peptides, HA9 Gag (left), NY10 Gag (middle), and VY/VF8-Nef (right) (top panels) and C-clade southern African subjects based on responses to OLPs containing the corresponding optimal peptides (bottom panels). In each case, a positive ELISpot response is defined as >100 SFC/10^6 PBMCs; *P* values are by Fisher's exact test (A, B, and C) (and for B and C are shown only when significant after correction for multiple comparisons) or by Mann-Whitney U test (D).

Gag NY10 is the single epitope differentially targeted by HLA-B*3501 subjects with B- and C-clade infection. Reactivity to the panel of HLA-B*3501-restricted epitopes defined was determined in HLA-B*3501-positive subjects with B-clade infection ($n = 74$) and in subjects with C-clade infection ($n = 42$) using ELISpot assays (Fig. 4). Overall, p24 Gag-specific epitopes were targeted significantly more frequently by the C-clade-infected B*3501-positive study subjects (55% versus 31%; $P = 0.02$ by Fisher's exact test), whereas Env-specific epitopes were targeted more frequently by B-clade-infected B*3501-positive study subjects (10% versus 26%; $P = 0.05$ by Fisher's exact test) (Fig. 4A). At the individual epitope level, the single statistically significant clade-specific difference was in the response to the Gag NY10 epitope (Gag positions 253 to 262; $P = 2 \times 10^{-5}$). A response to this epitope was seen in only 5% of B-clade-infected subjects, versus 38% of C-clade-infected subjects. Although the Rev epitope KY10 (Rev positions 14 to 23) was also predominantly targeted in C-clade infection, this difference in recognition in B- and C clade-infected HLA-B*3501-positive subjects did not reach statistical significance after correction for multiple comparisons.

Both p24 Gag responses and one Nef response are consistently associated with lower viral load in subjects with HLA-B*3501. Having determined which HLA-B*3501-restricted epitopes are targeted in B- and C-clade-infected subjects with HLA-B*3501, we next investigated which of these responses appear to be most effective in bringing about a low viral set point. Two responses were consistent in being associated with a lower set point in the responders compared to the nonresponders in both B- and C-clade cohorts, Gag HA9 and Nef VY8 (Fig. 4D). These two epitopes are targeted equally well in B- and C-clade infection, and therefore these responses do not help to explain why HLA-B*3501 is associated with lower viral set points in C-clade infection. In the case of Gag NY10, however, in B-clade infection there was only 1 responder among 31 B-clade subjects for whom viral loads were available. However, in the C-clade-infected cohort, a response toward Gag NY10 was also associated with a lowered viremia ($P = 0.03$ by Mann-Whitney test) (Fig. 4D), Thus, the only HLA-B*3501-restricted response associated with a lower viral set point for which there was a significant difference in epitope targeting comparing the B- and C-clade cohorts was the Gag NY10 response.

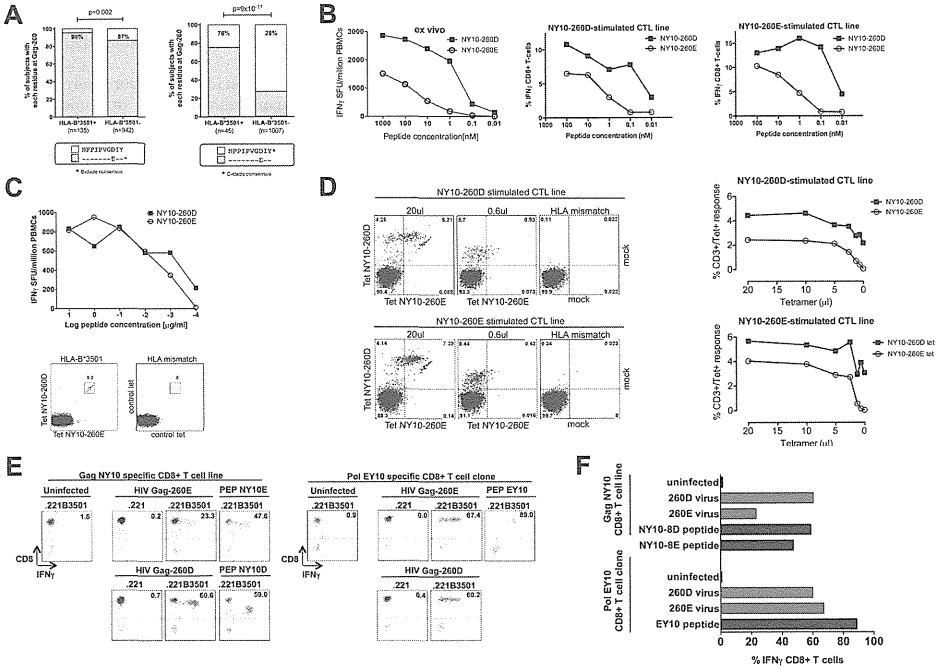

FIG 5 Selection of Gag-D260E substitution in C-clade infection and effect of this polymorphism on CD8$^+$ T-cell recognition and lack of NY10-260E specific CD8$^+$ T cells. (A) Selection of Gag-D260E polymorphism in subjects with HLA-B*3501 from an extended B-clade data set as previously published (24) (n = 1,077; total subjects with HLA-B*3501, n = 135 [12.5%]) (left) and selection of Gag-D260E polymorphism in subjects with HLA-B*3501 from an extended southern African data set (Durban, n = 695; Botswana, n = 298; Thames Valley Africans, n = 59; total subjects with HLA-B*3501, n = 45 [4.3%]) (right). (B) IFN-γ *ex vivo* ELISpot responses made by an HLA-B*3501-positive Japanese subject with chronic B-clade infection (subject KI705, HLA-A*2402, -A*2601, -B*3501, -B*5201, -Cw*0303, -Cw*1202) to optimal epitope NY10 (NPPIPVGDIY) and an escape variant containing the D260E substitution (NPPIPVGEIY) and IFN-γ intracellular cytokine staining of CD8$^+$ T cells *in vitro* expanded and tested against titrated amounts of NY10-260E and NY10-260D peptides. One experiments was performed. (C) IFN-γ *ex vivo* ELISpot responses made by an HLA-B*3501-positive subject with chronic B-clade infection (subject OX035, HLA-A*0201, -A*1101, -B*1801, -B*3501, -Cw*0401, -Cw*0501) to optimal epitope NY10-260D and an escape variant containing the D260E substitution NY10-260E and dual NY10-260E and NY10-260D HLA-B*3501 tetramer staining of *ex vivo* PBMCs controlled by HLA-B*4201 mismatch tetramer. Results from one representative of two independent experiments are shown. (D) *In vitro*-expanded PBMCs from subject OX035 using NY10-260D (top) and NY10-260E (bottom) peptides and stained with titrated amounts of dual HLA-B*3501 tetramers (260D/260E) gated on CD8$^+$ T cells (dot plots) and expressed as CD3$^+$/Tet$^+$ positive cells for all tetramer titrations (right) controlled by HLA-B*4201 mismatch tetramers. P values are by Fisher's exact test. One experiments was performed. (E and F) HLA-negative and HLA-B*3501-expressing target cells were infected with either Gag-260E or Gag-260D virus and tested for epitope recognition by specific CD8$^+$ T cells determined by IFN-γ production after coculture and shown for Gag-NY10 epitope processing (left) or the control Pol-EY10 epitope (right) by fluorescence-activated cell sorter (FACS) plots (E) and shown as horizontal bar graphs (F). Peptide-pulsed target cells (PEP) were included as a positive control for optimal epitope presentation.

Lack of immunogenicity of NY10-260E indicated by strong selection of the Gag-D260E polymorphism in B- and C-clade infection and lack of NY10-260E-specific CD8$^+$ T-cell responses. We next addressed the question of why the B-clade version of Gag NY10, which differs from the C-clade version only at position 8 in the epitope, in the replacement of Asp by Glu (Gag-D260E), appears to be nonimmunogenic, whereas the C-clade version is highly immunogenic. Although 38% of HLA-B*3501-positive subjects with chronic C-clade infection show detectable responses to NY10-260D, analysis of *gag* sequences in the cohort indicates that exactly twice that figure, 76%, of HLA-B*3501-positive subjects carry the Gag-D260E mutation, compared to 28% of the HLA-B*3501-negative study subjects (Fig. 5A) ($P = 9 \times 10^{-11}$). We confirmed that, in every case tested, the NY10-D260E variant is substantially less well recognized than the C-clade wild-type NY10-260D (Fig. 5B) and that NY10-D260E is therefore an escape mutant. Strikingly, NY10-260E is also selected in HLA-B*3501-positive subjects with B-clade infection (Fig. 5A), in spite of the fact that close to 90% of B-clade sequences carry Gag-260E (37). These data suggest that NY10-260E is nonimmunogenic and that only the small fraction of B-clade-infected HLA-B*3501-positive subjects presented with virus expressing the Gag-260D variant can make an NY10-260D-specific response, from which the viral escape mutant D260E is selected.

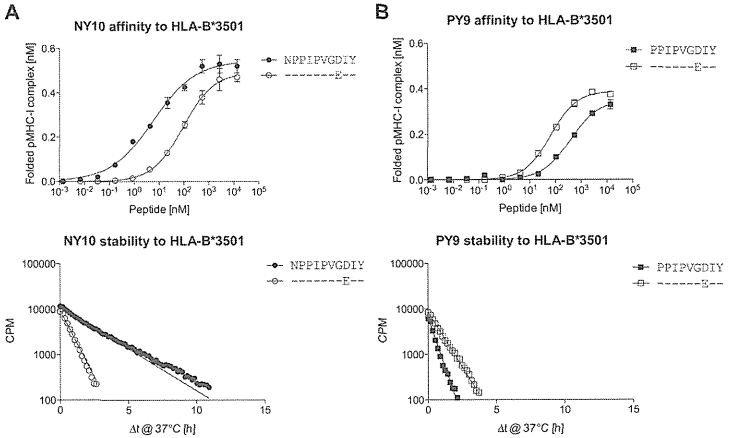

FIG 6 Binding of NY10 Gag and PY9-Gag to the HLA-B*3501 molecule. Strength of binding affinity (K_d, nM) of HLA-B*3501 was determined using the luminescent oxygen channeling immunoassay, as previously described (29) (top panels), and stability half-life ($t_{1/2}$) of binding (h) was determined using scintillation proximity assay, as previously described (30) (bottom panels), for NY10 (A) and PY9 (B). Results from one representative of four independent experiments are shown.

To test this hypothesis, i.e., that NY10 responses in B-clade-infected subjects are either cross-reactive between the two NY10-260D and NY10-260E variants or specific to the NY10-260D form but are never specific for the NY10-260E variant, we generated HLA-B*3501-NY10-260D and HLA-B*3501-NY10-260E tetramers with which to stain NY10-specific CD8$^+$ T cells. Staining of PBMCs and antigen-specific cell lines with these two HLA-B*3501-NY10 tetramers was consistent with the hypothesis (Fig. 5C and D). *In vitro* expansion of NY10-specific CD8$^+$ T cells in the rare B-clade-infected persons showing a response to this epitope showed, irrespective of which variant had been used to stimulate PBMCs, preferential recognition of the NY10-260D (C-clade version) of the epitope (Fig. 5B). Where there is apparent cross-reaction of NY10-260D-specific CD8$^+$ T cells to the NY10-260E variant (Fig. 5C), following *in vitro* expansion of these cells using either the NY10-260D or the NY10-260E peptide, preferential recognition of the NY10-260D epitope consistently emerges. Dual NY10-260D and NY10-260E tetramer staining confirms that only cross-reactive or NY10-260D-specific CD8$^+$ T cells exist, with no detection of NY10-260E-specific CD8$^+$ T cells (Fig. 5D). To test whether the 260E escape version has a reduced recognition compared to the 260D version using intracellular processed epitopes, rather than peptide-pulsed cells, we infected HLA-B*3501-positive or HLA class I-negative cells with HIV containing either the 260D or the 260E virus and determined the level of NY10 epitope recognition by assaying the activation of an NY10-specific CD8$^+$ T-cell line after coculture with cells infected for 6 days (Fig. 5E and F). We detected almost 3-fold-higher activation after infection with the 260D virus compared to the 260E virus (CD8$^+$/IFN-γ$^+$, 60.6% versus 23.3%) but equal activation of the control Pol EY10-specific CD8$^+$ T-cell clone. Thus, infection with the 260E virus results in a markedly reduced recognition of the nonimmunogenic NY10-260E compared to the immunogenic NY10-260D epitope processed from the 260D virus.

NY10-260E nonimmunogenicity results from lack of HLA-B*3501–peptide binding affinity and stability. Given that the peptide-binding motif for HLA-B*3501 does not show any preference for particular residues at position 8 (P8) in the epitope, our initial hypothesis was that nonimmunogenicity of the NY10-260E variant might be related to the low TcR repertoire available for HLA-B*3501-restricted T-cell responses, as proposed by Kosmrlj et al. (41). However, to determine whether that NY10-260E nonimmunogenicity might be more readily explained as a result of weak HLA-B*3501 binding affinity and/or stability, we first performed these MHC binding studies. We found that the immunogenic, NY10-260D (C-clade) version of the peptide had a >10-fold-greater binding affinity to the HLA-B*3501 molecule than the NY10-260E (B-clade) variant and was more than three times more stable in complex with the HLA-B*3501 molecule than the NY10-260E version (half-life, 1.6 h versus 0.5 h) (Fig. 6A). Previous studies suggest that, with rare exceptions, a peptide-MHC stability half-life of >1 h is required for peptides to be immunogenic (31). The low peptide-MHC binding stability of the NY10-260E variant (half life, 0.5 h) would therefore explain the lack of NY10-specific responses observed for the B-clade cohorts studied here. This is also consistent with reduced recognition of the NY10-260E versus the NY10-260D version of the epitope shown in Fig. 5.

It is noteworthy that had PY9 as opposed to NY10 been the optimal epitope in this case, it would not have been able to explain lack of immunogenicity of the B-clade variant in this way. Both B- and C-clade versions of PY9 had low peptide-binding affinities to HLA-B*3501, in particular the Gag-260D (C-clade) version (K_d = 76 and 407 nM for PY9-260E and PY9-260D, respectively), and very low peptide-B*3501 binding stabilities, again lower for the

Gag-260D C-clade version of PY9 (half-life of 0.62 h and 0.34 h for PY9-260E and PY9-260D, respectively).

Together these data suggest that the observed differential HLA-B*3501 association with HIV disease progression in B- and C-clade infection may hinge on a single Gag epitope, NY10, and that the lack of immunogenicity of this epitope in B-clade infection rests on the presence of Glu at Gag-260 in the consensus B-clade sequence, in contrast to Asp at Gag-260 in the consensus C-clade sequence.

DISCUSSION

The data presented here demonstrate that subjects with HLA-B*3501 control HIV-1 more effectively in C-clade than in B-clade infection. This difference was associated with greater targeting of p24 Gag epitopes and less frequent targeting of Env epitopes overall. However, the single epitope significantly targeted differentially was the Gag NY10 epitope, targeted by 38% of HLA-B*3501-positive subjects with chronic C-clade infection and only 5% of HLA-B*3501-positive subjects with chronic B-clade infection. The reason for this difference is the replacement of Asp by Glu at Gag-260, position 8 within the NY10 epitope: in C-clade infection, ~75% of sequences carry Asp at Gag-260, whereas in B-clade infection, ~90% of sequences carry Glu at Gag-260. NY10-260E is nonimmunogenic and insufficiently recognized from infected cells (<25% $CD8^+$ T-cell activation) because this variant fails to bind sufficiently stably to HLA-B*3501. In contrast, the NY10-260D version is recognized more efficiently (>60% $CD8^+$ T-cell activation) and binds relatively stably to HLA-B*3501 (off-rate half-life of 1.6 h, compared to 0.5 h for NY10-260E). The binding affinity of HLA-B*3501 for NY10-260D was also substantially higher than that for NY10-260E (K_d of 10 nM versus 113 nM, respectively), consistent with the difference in antigen processing of this epitope. These findings provide a plausible explanation for why NY10-260E is an escape variant in B- and C-clade infection and why only the NY10-260D variant is immunogenic.

Several hypotheses have previously been proposed to explain the rapid disease progression of HLA-B*3501-positive subjects infected with B-clade HIV, including a paucity of HLA-B*3501-restricted Gag-specific $CD8^+$ T-cell epitopes (39), failure to optimize antiviral NK activity (51, 52) and narrowness of the TcR repertoire available to counter epitope sequence variability (41). The data presented here support the "Gag hypothesis" (39), in that even the addition of a single extra Gag response appears to significantly alter the impact of HLA-B*3501 in HIV infection. This is consistent with previous findings that increasing Gag-specific $CD8^+$ T-cell breadth is correlated with increasing viral suppression (39) and that the p24 Gag protein is infrequently targeted by HLA-B*3501-restricted $CD8^+$ T-cell responses in B-clade infection (67). These data also support previous studies that have suggested that even one effective CTL response can mediate long-term immune control of immunodeficiency virus infection, such as the KK10 (Gag positions 263 to 272) response in HIV-infected subjects with HLA-B*27 (27) or the SW9 (Gag positions 241 to 249) response in simian immunodeficiency virus (SIV)-infected Burmese macaques expressing the MHC 90-120-Ia haplotype (35, 70).

These data show that inadequate HLA-B*3501 binding of the peptide, as opposed to TcR paucity, as has been proposed as a mechanism for HLA-B*3501-associated rapid progression (41), may provide the explanation for the lack of a response to NY10-260E in B-clade infection. The two hypotheses are not mutually exclusive, and it remains possible that HLA-B*3501 is associated with some degree of protection against C-clade progression in spite of TcR paucity. However, the distinction is of direct relevance to vaccine design, since we show here that HLA-associated disease outcome is dependent on the epitopes being targeted, irrespective of any deficiencies attributed to the respective HLA molecule. Furthermore, it is striking that HLA-B*0702 and HLA-B*3501, the two alleles proposed to predispose to rapid HIV progression as a result of TcR paucity (41), both have a more successful impact on the viral set point in C-clade infection (Fig. 1), as do many other alleles within the HLA-B7 supertype whose peptide-binding motifs are very similar, namely, HLA-B*8101, B*4201, B*0705, and B*3910 (45).

These studies also draw attention to caveats associated with epitope prediction approaches using peptide-binding motifs or even those using the most sophisticated software that takes account of the possible contribution to MHC binding of every amino acid of every peptide known to bind to a particular MHC class I molecule. Although PPIPVGDIY (PY9) has appeared in the "A" list of HIV-specific $CD8^+$ T-cell epitopes since 1995 (48) and epitope prediction programs predict that PY9 would bind better than NY10 to HLA-B*3501 (17), nonetheless PY9 is not the epitope. It is significant that 0/377 peptides eluted from HLA class I molecules and sequenced have Pro at P1 (44). Bearing in mind the specificity of ERAP-1, which cleaves neither at X-P nor at P-X bonds (32), it seems that epitopes carrying Pro at P1, if they exist at all, are rare. The importance of defining the precise optimal epitope correctly is underlined by this study, in the demonstration that the 10-mer NY10 could only be immunogenic with Asp at P8 (Fig. 5). In contrast, although PY9-260E bound with stronger avidity than PY9-260D to HLA-B*3501, neither version of the 9-mer PY9 appeared to bind HLA-B*3501 with adequate stability to be immunogenic. It may be helpful in the future to confirm the identification of novel epitopes using peptide-MHC I tetramers, as now can be done readily (40, 43).

The critical contribution to MHC binding of the residue at P8 in an HLA-B*3501-restricted epitope was unexpected, given the peptide-binding motif of HLA-B*3501, which describes proline at P2 and Tyr at PC as the primary anchor residues, with various residues less strongly preferred at P2, P3, P4, and PC (18, 33). Explanation of this awaits the solution of the crystal structure of the HLA-B*3501–NY10-260D complex. However, an HLA-B*3501-EBV epitope structure has been solved (71), and modeling the HLA-B*3501-NY10 structure based on these data suggests that Asp at P8 in the NY10 epitope indeed points into the groove (Fig. 7). The model suggests that replacement of Asp by Glu at P8 would lead to steric hindrance between the longer side chain of Glu and the side chain of Ala-150 in the MHC α2 helix. The resulting altered conformation of the peptide would explain the observed reduction in stability of NY10-260E (Fig. 6B). This is consistent with the reduced but detectable processing of the NY10-260E peptide (data not shown) and is directly explained by the reduced affinity to the MHC molecule and thereby suggests that the limiting step in processing of the NY10-260E peptide occurs when the fully trimmed epitope is loaded onto the HLA-B*3501 molecule by the peptide-loading complex. This reduction in processing of the NY10-260E epitope may be critical to distinguish immunogenicity, especially at low infection levels of primary $CD4^+$ T cells *in vivo*, in contrast to the higher multiplicity of infection used *in vitro* in this assay.

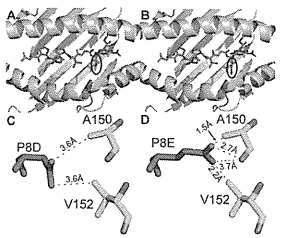

FIG 7 Modeled B3501-NY10 structure using the B3501-EPLPQGQLTAY complex (71). (A) HLA-B*3501 (shown in gray cartoon)-NPPIPVGDIY (shown in blue sticks), looking down at the MHC-binding groove. Position P8D in the peptide is circled. (B) HLA-B*3501 (shown in gray cartoon)-NPP IPVGEIY (shown in red sticks), looking down at the MHC-binding groove. P8E is circled. (C) Modeled interaction with NY10 residue P8D (blue stick) and MHC residues A150 and V152 (green sticks). (D) Modeled interaction with NY10 residue P8E (red stick) and MHC residues A150 and V152 (green sticks). The longer side chain of E in the escape mutant NY10 compared to D in the wild-type NY10 could generate steric hindrance with MHC residue A150. This could destabilize, and change the conformation of, the NY10 escape mutant peptide.

The high frequency (~75%) of the D260E selection in C-clade infection suggests a highly functional Gag NY10-specific $CD8^+$ T-cell response *in vivo*. However, when we undertook sequencing of the Gag NY10 region from position 253 to 262 of 17 HLA-B*3501-positive recipients with known Gag NY10 sequences of their linked donor viruses, we did not find any selection of D260E escape mutation at very early viral load set points (CD4 count nadir) in 10 HLA-B*3501 individuals infected with the 260D virus (0/10) (data not shown). This suggests that the D260E selection occurs after the CD4 nadir during chronic infection and that the Gag NY10 response therefore may operate during chronic infection rather than during acute infection. This is consistent with a previous study showing that the HLA-B*3501-D260E mutation is selected outside acute infection (54). Moreover, we did not observe any change in the viral load set point for individuals carrying 260D versus 260E within linked recipients (37,720 versus 39,740 RNA copies/ml plasma; $P = 0.6$) early after infection or during chronic infection (17,550 versus 26,600 RNA copies/ml plasma; $P = 0.58$) (data not shown). However, the small numbers in combination with the potential compensatory mutations identified, which may restore viral fitness, may mask differences in viral load set point.

Although the residue at Gag-260 appears to play an important part in immunogenicity of the HLA-B*35:01-NY10 epitope, it is also important to note that, as with many amino acid substitutions in p24 Gag, this single-amino-acid substitution at Gag-260 is often observed in association with a number of other variations elsewhere in p24 Gag. In a covariation analysis (14), we identified 9 statistically significant associations ($q < 0.05$) between Gag-260D and variation at other positions (see Table S3 in the supplemental material), which may indicate that the D260E escape in C-clade virus may require compensatory mutations to minimize the impact on viral replicative capacity.

One further observation with respect to epitope definition highlighted by this study is the value of using a panel of overlapping peptides to comprehensively map responses made by HIV-infected subjects, as opposed to using epitope prediction. The other p24 Gag epitope defined here in HLA-B*3501-positive subjects, HPVHAGPIA (HA9), may have gone unnoticed previously because HLA-B*3501 typically shows a binding preference for Tyr or a larger hydrophobic residue than Ala at the C terminus. Between 40 and 60% of subjects studied here with HLA-B*3501 made a response to HA9, and, like for the NY10 Gag response, responders had significantly lower viral loads than nonresponders. Thus, a critical epitope within p24 Gag would have remained undetected had we used an approach based on predicted epitopes only.

Of note, we unexpectedly showed that a response to one of the Nef epitopes, NY8, was also associated with a lowered viremia in both B- and C-clade infection. $CD8^+$ T-cell responses to Nef have not typically been associated with disease control (39), but the data presented here suggest that specific responses within Nef may also mediate viremic suppression. In a previous study, it was observed that a substantial number of the Nef escape mutations revert following transmission to an HLA-mismatched host (54), suggesting a cost to viral fitness; the escape polymorphism itself may therefore contribute to disease control via an effect on viral replicative capacity. This finding is also consistent with data describing effective control of SIV in Mamu-B*08- and Mamu-B*17-positive rhesus macaques, which tend to target dominant epitopes not in Gag but in proteins such as Nef and Vif (49, 57). Thus, although a broad Gag-specific $CD8^+$ T-cell response may be more likely to be effective against HIV, it remains possible that $CD8^+$ T-cell responses targeting epitopes in non-Gag proteins may also be effective in containment of immunodeficiency virus infection.

It is important also to consider the limitations of this study. In particular, attention should be drawn to the fact that optimal HLA-B*3501-restricted epitopes 8 to 11 amino acids in length were tested for recognition in the B-clade-infected Japanese study subjects, whereas the C-clade-infected subjects were tested for recognition of the 18-mer overlapping peptides containing those optimal epitopes. Although responses to the 18-mer and to the optimal epitope have been strongly correlated (16) ($r = 0.85$; $P < 0.0001$ [H. N. Kloverpris et al., unpublished data]), the magnitude of response to the 18-mer tends to be somewhat lower than that to the optimal epitope, particularly if the location of the optimal epitope is in the central part of the 18-mer peptide (16, 55). However, this likely underestimation of the responses in the C-clade-infected study subjects, where response frequencies were determined using the 18-mer overlapping peptides, would likely have reduced the estimates of the frequency of Gag NY10 responses and of Gag HA9 responses, detected in 38% and 52% of subjects, respectively. Therefore, the difference in targeting of p24 Gag epitopes that exists between B- and C-clade-infected subjects is likely, if anything, to be even greater than shown in Fig. 4.

In summary, the impact of HLA alleles such as HLA-B*3501 on HIV disease outcome differs according to clade of infection. These data suggest that the critical difference in C-clade infection is the ability of HLA-B*3501-positive subjects to make two p24 Gag-specific responses restricted by this allele, NY10 and HA9, compared to only one (HA9) in B-clade-infected subjects. This result provides the clearest data yet that HLA-associated disease outcome is dependent on the epitopes being targeted, irrespective of

the nature of the restricting HLA molecule (55), and this provides hope that a vaccine that can induce effective $CD8^+$ T-cell responses can successfully bring about immune control even in people who carry HLA alleles traditionally regarded as associated with rapid disease progression.

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We declare that no competing interests exist.

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INTERNAL MEDICINE

□ CASE REPORT □

Drug-Induced Acute Interstitial Nephritis Mimicking Acute Tubular Necrosis after Initiation of Tenofovir-Containing Antiretroviral Therapy in Patient with HIV-1 Infection

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Abstract

We describe a case of 68-year-old Japanese man with HIV-1 infection who developed acute kidney injury with prominent tubular dysfunction immediately after starting tenofovir-containing antiretroviral therapy. Antiretroviral therapy was discontinued in two weeks but renal function, as well as tubular function, did not shown full recovery even at a 3-year follow-up examination. Acute tubular necrosis, a rare but well-known side effect of tenofovir, was suspected, but kidney biopsy confirmed interstitial nephritis. It is important to distinguish drug-induced interstitial nephritis from acute tubular necrosis, because early steroid administration can improve renal dysfunction caused by acute interstitial nephritis.

Key words: tenofovir, acute interstitial nephritis, acute tubular necrosis, acute kidney injury, HIV infection, kidney biopsy

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Introduction

Renal proximal tubular dysfunction is a well-known side effect of tenofovir (1, 2). Although rare, it sometimes leads to acute tubular necrosis (ATN) and results in acute kidney injury (AKI) (1, 3). Drug-induced acute interstitial nephritis has a similar clinical presentation to ATN, but has different etiology and management (4, 5). Here we report a case of tenofovir-induced acute interstitial nephritis (AIN) which mimicked ATN after initiation of tenofovir-containing antiretroviral therapy (ART).

Case Report

A 68-year-old Japanese man with history of hypertension and diabetes mellitus was diagnosed with HIV infection and pneumocystis pneumonia (PCP). The latter was treated with sulfamethoxazole/trimethoprim plus prednisolone for three weeks, and the patient was referred to our hospital. Reactivation of PCP occurred and he was again treated with sulfamethoxazole/trimethoprim for three weeks. After completion of PCP treatment, sulfamethoxazole/trimethoprim was replaced with atovaquone for secondary prophylaxis, and one month later ART was started with tenofovir/emtricitabine plus lopinavir/ritonavir (baseline CD4 count 39/μL, HIV viral load 990,000 copies/mL). Baseline renal function tests were within the normal range (serum creatinine 0.53 mg/dL, blood urea nitrogen 8.7 mg/dL) with urine β2 microglobulin (β2MG) of 2,327 μg/L. The concurrent drugs were atovaquone (which was switched to prophylactic dose of sulfamethoxazole/trimethoprim on ART day 2), azithromycin 1,200 mg/week, and olmesartan. No concurrent nonsteroidal anti-inflammatory drug was used.

Serum creatinine started to rise and on ART day 14, it reached 2.66 mg/dL with β2MG of 321,400 μg/L. No fever or rashes were observed, but prominent eosinophilia was noted (18.6% of leukocytes, 4,400/μL). Urine dipstick test showed proteinuria +3, occult blood +2, and glycosuria +1, together with renal tubular epithelial cells and granular casts in urine. Serum potassium, sodium, and phosphate levels were within the normal ranges. Serum IgE was high (1,040 IU/mL), and serum antinuclear antibodies, antineutrophil cytoplasm antibody, and cryoglobulin were negative. Renal ultrasonography was also negative for specific findings.

ART and the other concurrent medications, with the exception of azithromycin, were discontinued on that day. Hydration with central venous catheter was started. At 21 days after commencement of ART, serum creatinine reached a peak level of 5.39 mg/dL, though renal function started subsequently to improve slowly. At 32 days after discontinuation of ART, ART with darunavir/ritonavir plus raltegravir was provided (serum creatinine 2.59 mg/dL). The patient was discharged 44 days after re-commencement of ART with a CD4 count of 247/μL, and HIV viral load of 2,700 copies/mL. Within 3 months after discharge, HIV viral load was suppressed to <50 copies/mL with a CD4 count of 316/μL.

Five months after the episode, renal biopsy was performed (serum creatinine 1.76 mg/dL, β2MG 15,677 μg/L). Examination of the specimen showed interstitial infiltration of lymphocytes, plasma cells, and a few eosinophils. There was no vacuolation in tubular cells and the brush border was intact. The glomeruli were histologically normal (Fig. 1a, b). Immunofluorescence study was negative for IgG, IgM, IgA, C1q, C3, C4, or fibrinogen. Electron microscopic examination demonstrated no abnormalities in the mitochondria of tubular cells (Fig. 1c). The final diagnosis was drug-induced AIN. Serum creatinine and β2MG were still elevated three years later at 1.47 mg/dL and 25,718 μg/L, respectively.

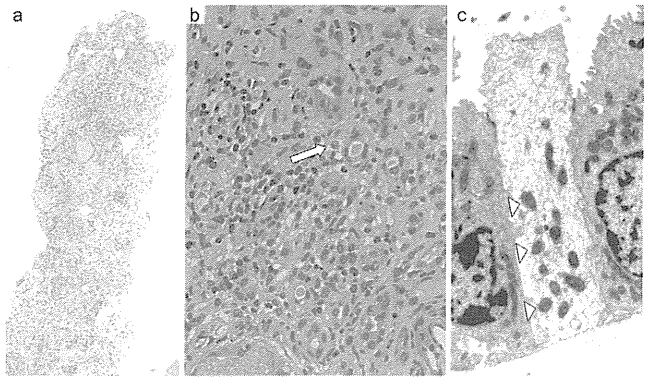

Figure 1. The microscopic findings in the renal biopsy specimen. (a) Diffuse interstitial inflammation with histologically normal glomeruli (Hematoxylin and Eosin (H&E) staining, ×10). (b) Prominent interstitial inflammatory infiltrates characterized by lymphocytes, plasma cells, and focal eosinophils (white arrow) (H&E staining, ×400). (c) Electron microscopic examination showed mitochondria normal in size and morphology in proximal tubular epithelial cells (white arrow heads) (×5,000).

Discussion

We described a case of tenofovir-induced AIN, which clinically mimicked ATN, after commencement of tenofovir-containing ART. Although the causative drugs were discontinued in two weeks, renal function did not show full recovery and the patient developed chronic kidney disease (Fig. 2). Tenofovir was highly likely the causative drug, because sulfamethoxazole/trimethoprim, the other drug which was used just before the occurrence of AIN, had been intermittently used for more than two months before the introduction of ART without any complications. To our knowledge, this is the fourth reported case of tenofovir-induced AIN, in addition to the three cases reported by Schmid et al. (6). Nevertheless, it is difficult to entirely role out the involvement of sulfamethoxazole/trimethoprim in occurrence of this AIN case. A combination effect of TDF and sulfamethoxazole/trimethoprim might have played a role.

It is difficult to diagnose interstitial nephritis based on clinical and laboratory findings only, and renal biopsy is required for a definitive diagnosis (4, 5). Only 5 to 10% of patients present with the classic triad of AIN symptoms: fever, rash, and eosinophilia (4, 5). However, renal biopsy is not performed in many cases with tenofovir-induced renal dysfunction, and thus, a considerable number of tenofovir-

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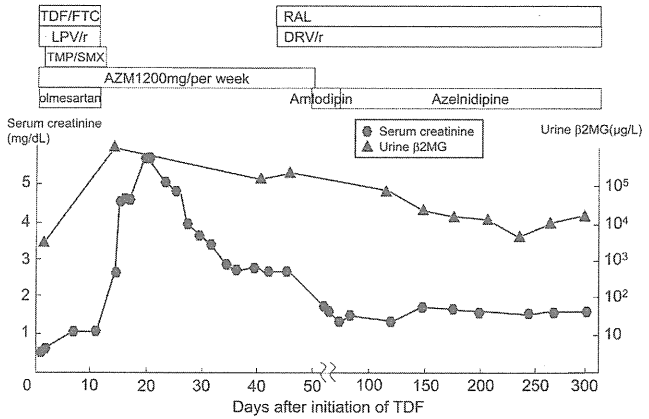

Figure 2. The clinical course of the patient. TDF/FTC: tenofovir/emtricitabine, LPV/r: ritonavir-boosted lopinavir, RAL: raltegravir, DRV/r: ritonavir-boosted darunavir, TMP/SMX: trimethoprim/sulfamethoxazole, AZM: azithromycin, β2MG: β2 microglobulin

induced AIN may have been misdiagnosed. Although a prominent eosinophilia and hyper-IgE (1,040 IU/mL) was noted for this case, these laboratory findings are commonly observed in patients with HIV-1 infection (7, 8). It is therefore difficult to dignose AIN solely based on these laboratory findings in patients with HIV infection.

The pathomechanism of tenofovir-induced ATN is considered to be mitochondrial toxicity in proximal tubular cells (9, 10). In contrast, interstitial nephritis occurs as an allergic response triggered by exposure to a drug (4, 5). It is important to distinguish AIN from ATN, because early steroid administration can improve the recovery of renal function in AIN (4, 5).

AIN should always be included in the differential diagnosis in a patient with AKI and prominent renal tubular damage following the introduction of tenofovir. In addition to prompt discontinuation of tenofovir, renal biopsy followed subsequently with steroid therapy at an early stage could produce a favorable renal outcome.

Author's disclosure of potential Conflicts of Interest (COI).
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All authors contributed to the concept, design, and writing of this submission.

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Blunted fetal growth by tenofovir in late pregnancy

Tenofovir disoproxil fumarate (TDF) is recommended for pregnant women coinfected with HIV and hepatitis B virus to prevent mother-to-infant transmission of both viruses [1]. However, the safety of TDF in pregnancy is still controversial, especially with regard to its effects on fetal growth and bone mineralization.

Here, we describe a 32-year-old HIV-1-infected Asian pregnant woman, who showed blunted fetal growth during TDF treatment. She had been treated during the first 33 weeks of pregnancy with abacavir, lopinavir/ritonavir and raltegravir based on multiple viral mutations. As plasma concentrations of raltegravir were persistently low, treatment was switched to TDF at 35 weeks of gestation, until delivery at 38 weeks. The fetal growth curves of biparietal diameters and femur length were within the normal ranges before starting TDF; however, the growth of both parameters was significantly blunted after starting TDF (Fig. 1). Furthermore, tubular reabsorption rates for phosphate, urinary β2-microglobulin and alkaline phosphatase were 88%, 2776 μg/L and 435 U/L, respectively, during the TDF-treatment period, compared with 97%, 140 μg/L and 182 U/L, respectively, during the non-TDF-treatment period. Plasma TDF concentration in the mother was 3536 ng/mL at 4 h after dosing and 776 ng/mL in cord blood. The infant was delivered by cesarean section without HIV-1 infection, and weighed 2218 g (−2 SDs for Japanese infants), with a height of 45.0 cm (−1.5 SD), and a head circumference of 29.5 cm (−2 SD). Furthermore, moderate tubular dysfunction was observed at birth (serum calcium: 7.4 mg/dL, serum phosphate: 4.6 mg/dL, alkaline phosphatase: 560 U/L, urine β2-microglobulin: 1780 μg/L), together with a high plasma TDF concentration [102 ng/mL at 24 h after delivery (28 h after mother's dosing)]. Although the body length was persistently short throughout the first 3 months (less than −2 SD), the hand X-ray at 1 and 3 months showed no signs of osteopenia or rickets.

The present case raises two concerns with regard to the safety of TDF in pregnancy. TDF can reduce bone mineral density [2]. Van Rompay *et al.* [3] reported that treatment of infant macaque with high-dose TDF caused proximal tubular dysfunction, growth retardation and osteomalacia. Our findings suggest that administration of TDF during pregnancy caused fetal disordered bone growth through modest proximal tubular dysfunction. However, it is not clear whether maternal TDF-associated tubular dysfunction will cause future bone growth retardation in infants. A large cohort study reported that significantly shorter height was observed at age 1 year in TDF-exposed infants [4], which is generally considered as one of the symptoms of mild rickets. However, in our case, despite the persistently shorter height of the infant (less than −2 SD) throughout the first 3 months of life, hand X-ray at 3 months showed no findings of osteopenia or rickets. Second, plasma TDF concentrations in our case were 10-fold higher than the previously reported values [5], probably reflecting the mother's small body size (weight: 47 kg), and consequently higher placental transfer of TDF to the fetus. Asian pregnant women, who have smaller body size, may impose a more severe effect on fetal growth retardation than that reported previously. The effect of TDF in pregnancy and its impact on fetal growth needs to be evaluated in more detail.

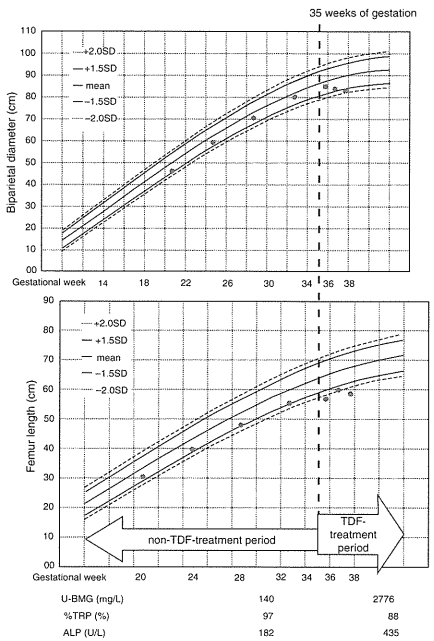

Fig. 1. Serial changes in fetal biparietal diameters, femur length and proximal tubular function during pregnancy. Between 20 and 33 weeks of gestation [non-tenofovir disoproxil fumarate (TDF)-treatment period], the growth curves of biparietal distance, femur length and proximal tubular function were within the normal ranges. However, the increases in biparietal distance and femur length were blunted after 35 weeks of gestation (TDF-treatment period), with a worsening of all markers of proximal tubular function. U-BMG, urine β2-microglobulin; %TRP, tubular reabsorption rate for phosphates; ALP, serum alkaline phosphatase.

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Conflicts of interest

There are no conflicts of interest.

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CASE REPORT

Raltegravir can be used safely in HIV-1-infected patients treated with warfarin

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Summary: Drug co-administration often affects the patient response to warfarin through various mechanisms. We describe here five HIV-1-infected patients on treatment with warfarin in whom the use of raltegravir was associated with a favourable outcome.

Keywords: HIV/AIDS, warfarin, raltegravir, etravirine, cytochrome P450, drug interaction, antiretroviral therapy

Drug co-administration often affects the patient response to warfarin through various mechanisms. For example, some drugs induce or inhibit liver enzymes, such as cytochrome P450 (CYP) isozymes responsible for warfarin metabolism;[1,2] others alter warfarin sensitivity by changing vitamin K synthesis or absorption, alter warfarin distribution or metabolism by increasing its affinity for receptor sites, or change the synthesis of functional coagulation factors. As the life expectancy of HIV-infected individuals is becoming longer, co-administration of warfarin with antiretrovirals needs to be assessed carefully. Nevirapine and lopinavir–ritonavir reduce serum concentrations of warfarin,[3,4] while efavirenz increases the concentration,[4] probably by the induction and inhibition of CYP2C9,[1,2] the main enzyme in warfarin metabolism. We reported previously the favourable effects of non-boosted fosamprenavir in patients treated with warfarin.[5] The clinical use of warfarin co-administered with raltegravir has not been described so far, though raltegravir seems to be a safe choice because it does not inhibit or induce CYP isoenzymes.[6] We describe here five HIV-1-infected patients on treatment with warfarin in whom the use of raltegravir was associated with a favourable outcome (Table 1). Cases 1–3 were Japanese men who had been treated with a stable dose of warfarin (mean daily dose, 3–4 mg) for underlying diseases, and their international normalized ratios (INR) were maintained within the optimal ranges (1.5–2.5 or 2.0–3.0) before the introduction of antiretroviral therapy (ART). Dose modification of warfarin was not necessary after starting ART containing raltegravir, as INRs remained within the optimal ranges. Case 4 was a 62-year-old Japanese man who had been treated with abacavir, lamivudine and non-boosted fosamprenavir (1400 mg twice daily). Based on his request, ART was switched to abacavir, lamivudine and raltegravir, and INR was maintained within the optimal range (1.5–2.5). Therefore, warfarin dose modification was not necessary. Case 5 was a 57-year-old Japanese man who had been treated with abacavir, lamivudine and lopinavir/ritonavir. He developed chronic atrial flutter. The initial dose of warfarin was 1 mg/day to maintain INR within the optimal range (1.5–2.5). Three months later, INR control became difficult at 4 mg/day of warfarin (INR; 0.70–0.91) and warfarin was terminated because it seemed ineffective. Non-boosted fosamprenavir could not be used because genotypic analysis showed resistance of HIV-1 to fosamprenavir. When raltegravir became available in Japan (9 months after discontinuation of warfarin), treatment was switched to ART comprising abacavir, lamivudine, raltegravir and etravirine, as well as warfarin (at initial dose of 1 mg/day). Three months later, INR was controlled within 1.46–2.49 at 3.5 mg of warfarin. The new regimen allowed maintenance of INR within the optimal range.

Cardiovascular events are increasing with the long-term use of ART. For patients treated with warfarin, raltegravir is a safe and clinically effective ART agent. Etravirine can potentially interact with warfarin by inducing CYP3A and mild inhibition of CYP2C9 and CYP2C19.[7] However, in Case 5, it was used successfully in combination with raltegravir. Such combinations may be helpful for the control of drug-resistant HIV-1 in warfarin-treated patients. Genetic polymorphisms in CYP2C9 may affect the response to warfarin,[8] though such data were not available in our five patients. The clinical introduction of raltegravir has expanded the ART options, though further clinical evidence is necessary in warfarin-treated patients.

Conflict of interest: None declared.

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Table 1 HIV-1 infected patients with favourable outcome following treatment with raltegravir and warfarin

No.	Age (years)	Sex	Underlying disease	ART regimen	Dose of warfarin (mean daily dose) (mg)	Maintained INR	Follow-up period (months)	Remarks
1	55	M	Chronic atrial flutter, cerebral embolism	RAL TDF FTC	3.5–4	1.69–2.64	15	–
2	57	M	Portal vein thrombosis	RAL ABC 3TC	3	1.68–2.31	2	–
3	59	M	Chronic atrial flutter, cerebral embolism	RAL ABC 3TC	3	2.03–2.94	3	
4	62	M	Chronic atrial flutter	RAL ABC 3TC	2.0–2.5	1.46–2.49	5	Switched non-boosted FPV to RAL.
5	57	M	Chronic atrial flutter	RAL ETV ABC 3TC	3.5	1.60–1.71	5	Switched LPV/RTV to RAL/ETV

RAL = raltegravir 800 mg/day; ETV = etravirine 400 mg/day; TDF = tenofovir 300 mg/day; FTC = emtricitabine 200 mg/day; ABC = abacavir 600 mg/day; 3TC = lamivudine 300 mg/day; FPV = fosamprenavir 2800 mg/day; LPV/RTV = lopinavir 800 mg/day and ritonavir 200 mg/day

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EASL JOURNAL OF HEPATOLOGY

Report from a Viral Hepatitis Policy Forum on implementing the WHO framework for global action on viral hepatitis in North Asia

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Background & Aims: The World Health Organisation (WHO) Prevention & Control of Viral Hepatitis Infection: Framework for Global Action offers a global vision for the prevention and control of viral hepatitis. In October 2012, the Coalition to Eradicate Viral Hepatitis in Asia Pacific (CEVHAP) organised the North Asia Workshop on Viral Hepatitis in Taipei to discuss how to implement the WHO Framework in the North Asia region. This paper presents outcomes from this workshop.

Methods: Twenty-eight representatives from local liver associations, patient organisations, and centres of excellence in Hong Kong, Japan, Korea, and Taiwan participated in the workshop.

Findings: Priority areas for action were described along the four axes of the WHO Framework: (1) awareness, advocacy and resources; (2) evidence and data; (3) prevention of transmission; and (4) screening and treatment. Priorities included: axis 1: greater public and professional awareness, particularly among primary care physicians and local advocacy networks. Axis 2: better economic data and identifying barriers to screening and treatment uptake. Axis 3: monitoring of vaccination outcomes and targeted harm reduction strategies. Axis 4: strengthening links between hospitals and primary care providers, and secure funding of screening and treatment, including for hepatocellular carcinoma.

Conclusions: The WHO Framework provides an opportunity to develop comprehensive and cohesive policies in North Asia and the broader region. A partnership between clinical specialists, primary care physicians, policy makers, and people with or at risk of viral hepatitis is essential in shaping future policies.

Keywords: Hepatitis B; Hepatitis C; Asia; Policy.
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Introduction

In 2012, the World Health Organisation (WHO) launched the *Prevention & Control of Viral Hepatitis Infection: Framework for Global Action*. This strategy offers a global vision for the prevention and control of viral hepatitis [1]. The Framework was welcomed by hepatitis experts and advocacy groups who have been struggling for the attention of policymakers about this 'silent epidemic' for many years [2,3].

Asia is home to 75% of all chronic hepatitis B cases [4] and China alone has more cases of hepatitis C infection than all of Europe or the Americas [5]. The majority of people infected with either hepatitis B virus or hepatitis C virus do not know that they are infected, and are not aware of the precautions they need to take to avoid infecting others or to enable them to reduce the impact of the infection [6]. Uptake of screening, when available, is low, and treatment rates are 4–10% in Asia compared to rates of 20% in the United States [7].

Against this background, the Coalition to Eradicate Viral Hepatitis in Asia Pacific (CEVHAP) was established in 2010 to contribute towards an Asia Pacific region free from the significant health, social and economic burden of viral hepatitis (www.cevhap.com). CEVHAP is uniquely positioned to support and facilitate the implementation of the WHO framework in different countries across the region through its network of members who are experts in their respective fields in the Asia Pacific region and globally.

In October 2012, CEVHAP organised the North Asia Workshop on Viral Hepatitis in Taipei, with participants from Hong Kong, Japan, Korea, and Taiwan. These four jurisdictions were chosen because, to varying degrees, they have some initiatives in place

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Table 1. Epidemiology of hepatitis B and hepatitis C in Hong Kong, Japan, Korea, and Taiwan.

	Hepatitis B				Hepatitis C		Hepatocellular carcinoma (HCC)			
Country [Ref.]	Prevalence of chronic hepatitis B infection, general population (%)	Estimated number of carriers ($x10^6$)	Age group with highest number of carriers	Prevalence in general population (%)	Dominant genotype	Time trends	Incidence in men; women (rate per 100,000 persons)	% due to hepatitis B infection*	% due to hepatitis C*	Median age of onset
Hong Kong [41]	8.8	0.7	>20 yr (prevalence increases with age)	0.30%	1b, 6a	Very low prevalence, most common in IDUs	29.9; 8.3	75-80	3-6 [44]	63 for men, 71 for women
Japan [12;20;42]	0.71	0.9	50-64 yr	0.63%	70% 1b, 20% 2a, 10% 2b	Risk factors changing over time and by region	2.42; 1	15	67.7	66.4 for men, 69.9 for women
Korea [43]	2.6	2.25-2.27	30-50 yr	1.29% (in >40 population)	1b, 2a	Mostly >40 age group people. Lack of data on youth, little data on role of injecting drug use	45; 33.6	20	72	Incidence increases after age 40, peak at 55
Taiwan [30;44]	10-12	2.5-3	35 (or 40)-55 (or 60) yr	4.4% (>20 yr)**	1b, 2a	Most disease in older groups. Significant geographic variations (from 0-90% depending on village) (45)	53; 21	53 [30]	28 (8% due to B + C) [30]	58 average, mean age 10 yr lower for HBV *vs.* HCV-caused HCC***

HCC, hepatocellular carcinoma; IDU, injecting drug users.
*The remainder of cases of HCC is caused by alcohol and other factors such as aflatoxin.
**This data is from populations participating in screening programmes only.
***One would expect the relative proportion of HCV-related HCC and the age of onset of HCC to increase in future.

in the area of viral hepatitis and have broadly similar health infrastructures. These localities are also in a privileged position compared to other countries in the Asia Pacific region, in that they have the resources to build on existing successes and lead the drive for further policy change across the region. Summary epidemiological data on hepatitis B and hepatitis C in these four jurisdictions is presented in Table 1.

The aim of the workshop was to ensure that participants understood the WHO framework; to support participants in building or strengthening advocacy networks, and to identify local priorities for implementing the framework within their respective jurisdictions.

This paper summarises the outcomes of this workshop and identifies steps to be taken to translate the WHO Framework into sustainable national policies on viral hepatitis in North Asia.

Materials and methods

The 28 workshop participants were identified within the existing CEVHAP network of local liver associations, patient organisations, and centres of excellence in Hong Kong, Japan, Korea, and Taiwan. The agenda for the one and a half day workshop was developed in close consultation with a small group of CEVHAP experts. To assist participants in their preparation, a briefing paper describing the scope of viral hepatitis, focusing on hepatitis C and hepatitis C virus, within the four jurisdictions was distributed prior to the meeting (CEVHAP, data on file).

The workshop used the four axes of the *WHO Prevention & Control of Viral Hepatitis Infection: Framework for Global Action* to guide discussions (Fig. 1) and consisted of expert presentations, group discussions, and country-level workshops.

Results

This paper uses the four axes of the WHO framework to describe the workshop results. The priority areas for action in the four participating jurisdictions are presented in Table 2 and are discussed in more detail in the section below.

Axis 1: Raising awareness, promoting partnerships, and securing resources

In North Asia, the general public, people at risk of infection, the medical community and policymakers generally have a poor understanding of viral hepatitis, its natural history and

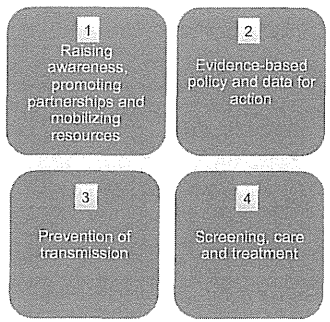

Fig. 1. The four strategic axes for policy development recommended in the WHO Prevention & Control of Viral Hepatitis Infection: Framework for Global Action.

manifestations. Awareness among primary care physicians is particularly low and targeted educational efforts are needed to encourage these providers to test their patients for viral hepatitis and refer them towards appropriate care pathways. Investment in developing better relationships between primary care and hepatitis specialist services may help engage primary care physicians.

Local advocacy networks that bridge civil society, liver specialists, primary care physicians and other community care providers are still lacking in Taiwan, Hong Kong, and Korea particularly. This lack of a strong advocacy base makes it more difficult to engage the media in the first place or to overcome media fatigue about viral hepatitis. The media plays a vital role in raising awareness of viral hepatitis, particularly among the general public and those at risk of infection. The awareness campaigns run in the United States and Korea provide interesting examples of media engagement on viral hepatitis (Case studies 1 and 2).

A key to the success of awareness campaigns on viral hepatitis is to find the issues that resonate best with media, the public, and policymakers. The fact that viral hepatitis is one of the main causes of liver cancer is indeed compelling and one with potential to grab the attention of these key stakeholders. For example, a recent study by the International Agency for Research on Cancer showed that one in six cancers was caused by infection and concluded that prevention of viral hepatitis and other infections could have a substantial effect on reducing the future burden of cancer [8]. These data may be very powerful in convincing policymakers of the need to mobilise resources towards the prevention and management of viral hepatitis.

Table 2. Priorities for action in Hong Kong, Japan, Korea, and Taiwan according to the four strategic axes of the WHO Global Framework.

Priorities for action
1. Raising awareness, promoting partnerships and mobilizing resources
Greater public awareness
Greater awareness of primary care physicians
Building patient advocacy
Strengthening hospital-primary care networks
2. Evidence-based policy and data for action
Economic data on the burden of viral hepatitis
Better data on barriers to screening and treatment
Centralised surveillance
Accurate estimates of the number of chronic hepatitis cases
3. Prevention of transmission
Better monitoring of vaccine effectiveness
Universal vaccination of children and improved access to vaccination by people at greater risk
Targeted harm reduction strategies
Better data on vaccine failure
4. Screening, care and treatment
Improved availability and funding of screening [public funds and/or employer-based]
Linking screening to effective monitoring and treatment
Funding screening for hepatocellular carcinoma
Improved access to treatment of chronic hepatitis and hepatocellular carcinoma

Case Study 1: How to engage the public on hepatitis: the 'KNOw More Hepatitis' in the United States

In 2011, the United States Centers for Disease Control and Prevention (CDC) launched an education campaign, 'KNOw More Hepatitis' [9]. Insights from focus groups consisting of people with high prevalence rates of infection (for example, 'baby-boomers' for hepatitis C) helped guide the development of targeted messages for each risk population [10]. The campaign made creative use of social and other media:

- It used powerful, evidence-based messages to engage the media. One example was "Hepatitis now kills more Americans than HIV", which was the key conclusion of a recently published article in the *Annals of Internal Medicine* [11].
- An online hepatitis risk assessment tool was featured on the CDC website, which allowed individuals to conduct a quick, confidential assessment of their risk for hepatitis A, hepatitis B or hepatitis C in the privacy of their own homes.
- The campaign has an active Facebook page, 11,000 followers on Twitter, and public service advertisements on YouTube. 400 tweets translated into over 3.3 million media impressions, demonstrating the power of social media to engage target audiences on viral hepatitis.
- Six national airports donated space worth up to $4 million for Dioramas which featured rotating posters on viral hepatitis (Fig. 2).

Case Study 2: Conveying the 'right level of fear'? The Korean experience

In March 2011, the Korean Association for the Study of the Liver (KASL) launched an awareness campaign on viral hepatitis. A 30-minute television advertisement showed patients with end-stage liver disease. The message was: "if you don't manage your disease, this is what is going to happen." The goal was to shock the public into action.

The impact of the advertisement was significant: the day after it featured, KASL was ranked top of Google searches. But the increased attention also had unintended adverse consequences: people infected with viral hepatitis reported the loss of relationships or employment as a result of the advertisement. KASL immediately launched a lower-intensity campaign that focused on the importance of seeking proper care for chronic hepatitis infection.

The lesson learned by KASL was that it is important to convey the 'right' level of fear about viral hepatitis in order to raise awareness of the urgency of the situation in terms of the risks of advanced liver disease. However, too much fear may create panic and inertia, if the perceived message is that nothing that can be done to improve the outcomes of people with the viral hepatitis or that policy makers, physicians, and the public are powerless to effect change.

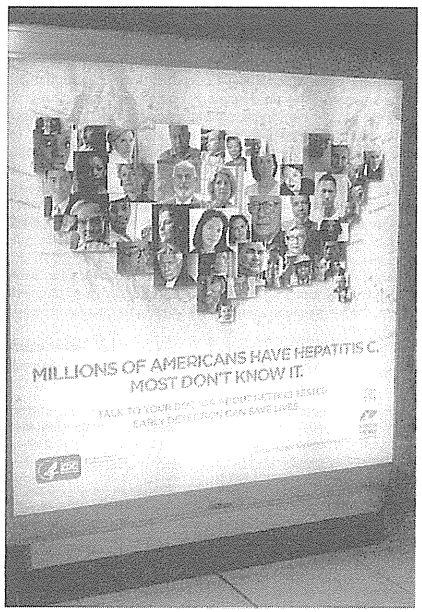

Fig. 2. Example of a diorama on viral hepatitis at a US airport.

Axis 2: Evidence-based policy and data for action

One key condition for successful advocacy and a sustained public health response is reliable data. With viral hepatitis, the fact that so many people remain undiagnosed makes it difficult to convey to policy makers the full scale of the problem [12]. Better surveillance is needed to capture chronic as well as acute cases of viral hepatitis. More reliable prevalence estimates in high risk populations, such as people who are poor, those who inject drugs, prisoners, and sex workers, are needed as these groups are usually poorly represented in existing surveillance studies.

Reliable economic data are critical to demonstrate to national governments the need for them to invest in viral hepatitis prevention and control. Sometimes showing policy makers the cost of 'doing nothing' can exemplify the most compelling case for investment [13].

One area where more research is greatly needed is to find the barriers to uptake of screening and treatment among individuals at risk. These data are critical to shift the behaviours of individuals towards more active disease management.

Finally, insights from patients, such as those gathered in a survey of the Japan Hepatitis Council (Case study 3) may help channel efforts towards areas that will make the greatest difference to individuals living with viral hepatitis.

Case Study 3: The combined power of advocacy and data: The Japan Hepatitis Council

Japan has a powerful patient advocacy base consisting of over 80 local, regional and national associations acting under the umbrella of the Japan Hepatitis Council. Pressure from these groups over the government's failure to implement blood and mass vaccination safety measures was instrumental in the creation of the Basic Act of Hepatitis Measures in 2010. As part of this Act, each prefecture is required to have a hepatitis patient representative on its local council.

A recent survey of members of the Japan Hepatitis Council helped identify some of the main challenges for policy development in Japan [14]:

- **High mortality from hepatocellular carcinoma (HCC):** Japan has one of the highest rates of HCC in the world and counts 30,000 deaths due to HCC every year.
- **Low uptake of screening:** A national screening programme against hepatitis B and C has existed since 2002, targeting individuals aged 40-70 years. However, uptake rates remain low (7-27%) and screening is poorly integrated into general practice [15, 16].
- **Poor linkage to treatment:** 48% of those who test positive for hepatitis B (and 65% of those testing positive for HCV) fail to seek medical care [12] and only half of those with hepatitis C who do seek care complete their course of treatment [14].
- **High costs of care:** Government funding for antiviral treatment of hepatitis B and hepatitis C has gradually increased since 2008, however patients are still left with a significant co-payment and many patients report crippling personal economic costs.
- **Stigma and discrimination:** Thirty percent of respondents report having experienced discrimination due to viral hepatitis, especially in medical institutions. Several respondents felt that their hepatitis status hindered their marriage prospects and employment options. Many admitted that they hid their condition from others as a result.

Axis 3: Prevention of transmission

Vaccination against hepatitis B has had a marked impact on reducing the incidence of hepatitis B infection (Case study 4). However, gaps in the region remain. Japan only offers vaccination to infants born to hepatitis B-infected mothers, whereas in Taiwan this is one group in whom vaccination efforts have been less successful. In all countries, careful evaluation of the impact of vaccination and of the benefits of extending vaccination to high risk groups is needed.

Injecting drug use is now the predominant route of transmission for hepatitis C in north Asia [17] and this is a critical target group for prevention strategies. Co-infection of hepatitis B and hepatitis C and/or HIV is a key concern in people who inject drugs, as it is associated with more rapid progression to liver disease and death [18,19]. Targeted education and prevention measures, including vaccination, are needed to control transmission in other individuals at high risk of infection, including people who have tattoos and acupuncture, women of childbearing age, men who have sex with men, and prisoners. And continued education about the risks of transmission through sexual contact and the need for safe sex practices is needed for the general population.

Re-use of needles and syringes in medical practice is common practice in Asia and nosocomial spread of hepatitis C has been observed in outpatient clinics [20] as well as dialysis units [21–23]. Information about safe injection practices and the prevention of transmission should be essential components of professional education efforts.

Case Study 4: Taiwan: a vaccination success story

Taiwan launched one of the first universal vaccination programmes against hepatitis B in 1984 and the programme is heralded around the world as a true success story [24, 25]. Today, systematic vaccination is offered to all newborns, health workers and schoolchildren who missed the neonatal vaccination (catch-up vaccination). The impact of the programme on seroprevalence levels has been considerable (Fig. 3) and horizontal transmission amongst children decreased [26]. The HCC incidence among children has been significantly reduced, making the hepatitis B vaccine the first effective vaccine for the prevention of cancer [27]. The programme has also provided important insights into the natural history of hepatitis B, for example about the duration of conferred immunogenicity and the potential need for booster vaccinations [28].

Complacency must be avoided, however, as thousands of deaths due to viral hepatitis still occur every year in Taiwan. Prevalence rates have not decreased in adults [29] and the impact of vaccination is much lower in rural areas than in urban centres [28, 30]. Also, the success of vaccination cannot be taken for granted: diligent, continuous monitoring of the quality of available vaccines and of the outcomes of vaccination programmes is needed for the public health impact of the vaccination programme against hepatitis B virus to continue in Taiwan [31, 32].

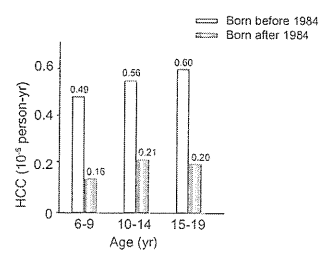

Fig. 3. Incidence of HCC by age in cohorts born before and after infant vaccination program against hepatitis B virus in Taiwan (started in 1984)[27].

Special Report

Axis 4: Screening, care, and treatment

Greater availability, awareness and uptake of screening for both hepatitis B and hepatitis C were highlighted as the most pressing needs by participants from all countries in the CEVHAP workshop. Countries differ in what screening programmes have been implemented and to what extent screening is covered by public funds. Barriers to screening are likely to be specific to each local context, not to mention each individual (Table 3). It is critical that the confidentiality of screening results is ensured; in many countries, the results of screening may be sent to a person's employer, causing discrimination and often loss of employment for the person concerned.

Another significant issue is the need to ensure greater linkage from screening to treatment, given a large proportion of individuals who test positive at screening are known not to seek treatment. Comprehensive care models are urgently needed to make sure that individuals who are infected receive appropriate information, counselling, and care throughout all phases of their condition [33]. In many countries, better collaboration between primary care physicians and liver specialists is needed to ensure that individuals who test positive are referred to appropriate care.

A commonly cited barrier to treatment was lack of public funding. Overall, government funding for antiviral therapies for both hepatitis B and hepatitis C has improved considerably over the past decade in all four jurisdictions (see Case study 5). However, out-of-pocket costs are often still high for many patients, be it for diagnosis, monitoring tests [21,34], or antiviral therapies. Funding of antiviral therapies in some countries is often limited to a given number of years, which may impact on compliance with long-term treatment regimens.

It is also important to recognise that lack of funding may sometimes be used as an excuse for not offering available treatments to patients. In truth, physicians are often unaware of existing treatment options, or they remain unconvinced of their benefit despite their inclusion in clinical guidelines and thus adopt a 'watch and wait' approach to treatment.

Table 3. Barriers to screening linked to individuals, providers and the healthcare system.

Source of barrier	Barriers
Individuals	Unaware that one is at risk of viral hepatitis Unaware that the disease can have serious long-term effects Unaware that effective treatments exist Cultural beliefs Stigma associated with viral hepatitis Costs associated with testing [lack of funding]
Health care providers	Social stigma Poor understanding of the availability and effectiveness of treatment Lack of disease management approach - 'wait and see' attitude to viral hepatitis Cost barriers to access treatment Lack of awareness about the need for monitoring [hepatitis B]
Healthcare system	Lack of continuity/no linkage from screening to care Cost of therapy/lack of government reimbursement

Adapted from [38].

Case Study 5: The importance of secure government funding for the treatment of viral hepatitis in Hong Kong

The Hong Kong government has funded antiviral therapy for hepatitis B and C since 2009, supported by annually renewable funding of approximately HKD 100 million. In 2010, an additional annually renewable HKD 76 million fund was set up for hepatitis B, with an estimated 3000 to 4000 extra patients receiving treatment. Funding for treatment is provided to hospitals as a prospective sum. Most of the funding has gone towards hepatitis B as the number of patients with hepatitis B infection is overwhelmingly greater than those with hepatitis C infection.

This secured funding has meant that patients with hepatitis B infection are offered guaranteed funding for their treatment without any limit as to its duration, which in Hong Kong practice, means nucleos(t)ide analogue treatment for life. Physicians claim this funding has transformed their relationship with their patients. Previously, patients would resist the prescription of long-term therapy for hepatitis B due to the financial burden it posed on them. Compliance was a significant problem. Since the changes in funding, the willingness to embark on life-long treatment has increased and compliance rates have improved significantly in patients with chronic hepatitis B infection in Hong Kong.

Experts believe that it was the demonstration of the cost-effectiveness of existing treatments that helped secure the funding, as well as the existence of two regular forums on hepatitis, the Scientific Working Group on Viral Hepatitis Prevention, and the Center for Health Protection, which offer an opportunity for governments to consult with leading liver specialists and for experts to present data to policy makers to help guide policy decisions.

Discussion

Medical science and public policy have reached a critical, and exciting, juncture for viral hepatitis: 179 countries worldwide have implemented vaccination programmes against hepatitis B. Up to 95% of cases of hepatitis B infection are now treatable and up to 60% of those of hepatitis C infection are curable [27,35,36]. Cirrhosis can be reversed [37] and treatment of liver cancer, once thought to be impossible, is now possible. Yet three-quarters of those infected with hepatitis B virus and 65% of those infected with hepatitis C virus do not know they are infected [3]. Screening uptake is low, as is uptake and adherence to treatment, with the result that outcomes for individuals infected with viral hepatitis remain suboptimal.

The CEVHAP North Asia Workshop on Viral Hepatitis highlighted the key challenges facing Hong Kong, Japan, Korea, and Taiwan in their fight against viral hepatitis. These challenges are similar to those in other regions [2,3]. The WHO Framework provides a blueprint for action, but the onus is on governments to reduce the burden posed by hepatitis locally, within the constraints and possibilities of their local epidemiology, resources, health care infrastructure, and advocacy base.

The research community has an important role to play in guiding policy development on viral hepatitis. Liver specialists, in partnership with voluntary sector organisations, may help ensure that key facts about viral hepatitis – for example, that hepatitis B is treatable and hepatitis C is curable – are communicated to the media, the public and policymakers in a way that is accessible and compelling. Social research and observational studies may help create a better understanding of the health seeking behaviours of people at risk of viral hepatitis and identify existing barriers to screening, diagnosis, and proper treatment.

The WHO Framework provides a unique opportunity to countries around the world to take stock of how they have addressed the challenges posed by viral hepatitis in the past and create comprehensive, cohesive policies that may have a lasting impact. This will require a collaborative effort from primary care physicians, specialists, governments, individuals at risk and people living with viral hepatitis. Working in partnership with other more high-profile disease areas, for example non-communicable diseases, may present opportunities to raise the profile of viral hepatitis. Indeed, lessons may be learned from other disease areas – such as breast cancer, cardiovascular disease and HIV/AIDS – which have raised awareness, secured funding and developed comprehensive policies that have changed the lives of people living with the condition. The WHO Framework provides the steer to do the same for the millions of people worldwide infected with viral hepatitis.

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Addendum

Participants of the Coalition to Eradicate Viral Hepatitis in Asia Pacific [CEVHAP] North Asia Workshop on Viral Hepatitis included: from Taiwan: Ding-Shinn Chen, Pei-Jer Chen, Sheng-Nan Lu, Pei-Ming Yang; from Hong Kong: Joseph Sung, Ching-Lung Lai, James Y.Y. Fung; from Korea: Si Hyun Bae, June Sung Lee, Hong Soo Kim, Sang-Hoon Ahn, Goo Hyeon Yoon; from Japan: Junko Tanaka, Takaji Wakita, Hideki Aizaki, Atsuko Yonezawa, Yukio Lino, Yoichi Abe; from the United States: John Ward, Lily Lou; from the UK: Charles Gore; from Malaysia: Rosmawati Mohamed; from Australia: Stephen Locarnini and Jack Wallace. The workshop was facilitated by Suzanne Wait (UK) and Jennifer Johnston (Australia).

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HEPATOLOGY

Characteristics of elderly hepatitis C virus-associated hepatocellular carcinoma patients

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Key words
alanine aminotransferase (ALT), alpha-fetoprotein (AFP), average integration value of ALT, elderly patient, hepatitis C virus (HCV), hepatocellular carcinoma (HCC), platelet count, propensity score.

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Abstract

Background and Aim: The average age of hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) patients has been rising in Japan. We evaluate characteristics of HCV-positive patients who develop HCC in older age to determine an optimal surveillance strategy.

Methods: A total of 323 patients with three or more years of follow-up before HCC diagnosis and 323 propensity-matched controls without HCC were studied. HCC patients were classified into four groups according to age at the time of HCC diagnosis: group A (≤ 60 years, $n = 36$), group B (61–70 years, $n = 115$), group C (71–80 years, $n = 143$), and group D (> 80 years, $n = 29$). Clinical and laboratory data were compared.

Results: Platelet counts were significantly higher in the older groups at HCC diagnosis ($P < 0.0001$). The rate of platelet counts decline was lower in older groups ($P = 0.0107$). The average integration value of serum alanine aminotransferase (ALT) in groups A, B, C, and D were 80.9 IU/L, 62.3 IU/L, 59.0 IU/L, and 44.9 IU/L, respectively ($P < 0.0001$). In older patients (≥ 65 years old), cirrhosis and average integration value of ALT were significantly associated with hepatocarcinogenesis, but platelet count was not.

Conclusion: Elderly HCV-positive patients (≥ 65 years old) with low ALT values developed HCC regardless of their platelet counts. These findings should be taken into account when designing the most suitable HCC surveillance protocol for this population.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies, particularly in southern and eastern Asia. In Japan, HCC is the third leading cause of cancer death in men, behind lung and stomach cancer. In women, HCC is the fifth leading cause of cancer death during the past decade, behind colon, stomach, lung, and breast cancer.[1] Hepatitis C virus (HCV) infection accounts for approximately 75–80% of cases. Each year, HCC develops in 6–8% of patients with HCV-associated cirrhosis.[2]

In Japan, screening the blood supply for HCV, which commenced in November 1989 and began using second-generation enzyme immunoassays in February 1992, decreased the risk of post-transfusion hepatitis from more than 50% in the 1960s to virtually zero presently.[3] The age of Japanese patients diagnosed with HCC has been steadily increasing. Up to 1999, the majority of HCC mortalities occurred in patients under 69 years of age, but in 2000 more than half of HCC patients were over the age of 70.[1] This aging trend is also observed in HCV patients undergoing interferon-based therapy in Japan.[4] In contrast, HCV infection in the United States and other western countries is most prevalent among persons 30 to 50 years of age,[5] and the incidence of HCV-associated HCC is expected to rise. As a country with more experience with HCV-associated HCC, Japan's long-term experience can be helpful in planning strategies to contain HCV infection and to cope with its long-term sequelae worldwide.

The aim of this study is to evaluate characteristics of HCV-positive patients who develop HCC in older age and to determine an optimal surveillance strategy for these patients.

Materials and methods

Study population. This study cohort was comprised of 6740 consecutive HCV-positive patients (1019 patients with HCC and 5721 patients without HCC) referred to the Department of Gastroenterology at Ogaki Municipal Hospital from January 1990 to December 2006.

There were 323 patients who fulfilled the following inclusion criteria out of 1019 HCC patients: (i) detectable HCV-RNA for at least six months, (ii) no evidence of hepatitis B virus infection; (iii) other possible causes of chronic liver disease were ruled out

(no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (iv) a follow-up period of greater than three years before HCC diagnosis; (v) no interferon therapy within the last 12 months; and (vi) serum alanine aminotransferase (ALT) measurements taken more than twice yearly. The patients were classified into four groups according to age at the time of HCC diagnosis: group A (≦ 60 years, $n = 36$), group B (61–70 years, $n = 115$), group C (71–80 years, $n = 143$), and group D (> 80 years, $n = 29$).

Of the 5721 patients who have not developed HCC, 3275 patients fulfilled the same inclusion criteria. To reduce the confounding effects of covariates, we used propensity scores to match HCC patients with unique control patients based on age, sex, Child-Pugh classification at the start of follow-up, and follow-up duration. We were able to match 323 patients with HCC to 323 patients without HCC. The patients were classified into four groups according to age at the end of follow-up: group A' (≦ 60 years, $n = 30$), group B' (61–70 years, n = 114), group C' (71–80 years, $n = 136$), and group D' (> 80 years, $n = 43$).

The start of follow-up was defined as the date a patient first visited our hospital and ended on the date of HCC diagnosis for the HCC patients, or the date of the last visit at our hospital or December 31, 2010, whichever occurred earlier, in control patients.

Histological examinations were performed in 234 out of 646 patients. Cirrhosis was diagnosed pathologically in 120 patients. The remaining 412 patients were evaluated with ultrasonography (US) and biochemical tests.[6–8] Patients who did not satisfy the criteria for cirrhosis were classified as having chronic hepatitis for the purposes of this study. All together, 288 out of 646 patients were diagnosed with chronic hepatitis, and 358 were diagnosed with cirrhosis.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital in January 22, 2009 and complied with the Helsinki Declaration. Each patient provided written informed consent.

Laboratory test for liver disease and virologic markers. Platelet counts, prothrombin time, and serum levels of ALT, albumin, total bilirubin, alpha-fetoprotein (AFP), *lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%), and des-γ-carboxy prothrombin (DCP) were determined at the start of follow-up. ALT is expressed as an average integration value.[6] Serum AFP concentration was determined with a commercially available kit. AFP-L3 was measured by lectin-affinity electrophoresis and antibody-affinity blotting with the AFP Differentiation Kit L (Wako Pure Chemical Industries, Ltd, Osaka, Japan).[9] DCP was quantified with the Picolumi PIVKA-II kit (Eisai Co., Ltd, Tokyo, Japan).[10] HCV genotype was determined by PCR using genotype-specific primers, and HCV-RNA was quantified (before November 2007: COBAS Amplicor HCV monitor test and after December 2007: COBAS AmpliPrep/COBAS TaqMan HCV test, Roche Diagnostics K.K., Tokyo, Japan).

Alcohol exposure. Past alcohol exposure was estimated based on chart review of drinking patterns over five years. Patients were categorized as either "excessive" or "moderate" alcohol consumers. Excessive alcohol consumers drank over 50 g daily for five years.

Methods of follow-up. All patients received medical examinations at least every six months at our institution. Imaging studies, either US, computed tomography (CT), or magnetic resonance imaging (MRI), were performed at least every six months. When patients were considered to have developed cirrhosis by laboratory data or imaging findings, imaging was performed at three-month intervals.[11]

Diagnosis and treatment of HCC. The diagnosis of HCC was made based on either pathological or clinical and radiological criteria. Histological examination of resected hepatic tumors or US-guided needle biopsy specimens confirmed HCC in 165 patients (resected specimens: 111 patients; biopsy specimens: 54 patients). In the remaining 158 patients, the diagnosis of HCC was made using clinical criteria and imaging findings obtained from B-mode US, CT, MRI, and CT angiography.[12,13]

Tumor staging was performed according to the American Joint Committee on Cancer (AJCC) classification system.[14] In cases where pathologic evaluation was not available, vascular invasion was assessed by dynamic CT and angiography.

Treatment for each patient was individualized according to evidence-based clinical practice guidelines for HCC in Japan.[14] Hepatic resection was performed on 111 patients. Percutaneous ethanol injection therapy was performed in 16 patients. Radiofrequency ablation therapy was performed in 104 patients. Transcatheter arterial chemoembolization was performed in 62 patients. Thirty patients did not undergo treatment because of the patient's wishes or impaired liver function.

Statistical analyses. Statistical analysis was performed with the Statistical Program for Social Science (SPSS ver.18.0 for Windows; SPSS Japan Inc., Tokyo, Japan). Continuous variables are represented as medians (range). The non-parametric Jonckheere–Terpstra test was used to assess continuous variables. The Steel–Dwass or Shirley–Williams multiple comparisons method was applied if the Jonckheere–Terpstra test yielded significant results. The Cochran–Armitage test or the chi-square test was used to assess categorical variables. Actual survival was estimated using the Kaplan–Meier method,[15] and differences were tested with the log-rank test.[16] The Cox proportional hazards model and forward selection method were used to estimate the relative risk of HCC development associated with age, sex, cirrhosis, alcohol consumption, diabetes mellitus, effect of prior interferon therapy, platelet count, AFP at the start of follow-up, and average integration value of ALT, and the annual rate of platelet count decline. Statistical significance was set at $P < 0.05$.

Results

Clinical features at baseline. The clinical profiles of the HCC patients at the start of follow-up are shown in Table 1. There was a higher proportion of women diagnosed with HCC at a later age ($P = 0.0016$); the percentage of women in groups A, B, C, and D was 13.9, 37.4, 44.1, and 51.7, respectively. As the patient's age at HCC diagnosis increased, the patient's age at the start of follow-up and the duration of the observation period until HCC diagnosis increased ($P < 0.0001$ and $P = 0.0003$, respectively). Patients who received a diagnosis of HCC at a more advanced age have a significantly decreased incidence of diabetes mellitus and prior interferon therapy ($P = 0.0008$ and $P < 0.0001$, respectively). The clinical profiles of the control patients at the start of follow-up are shown in Table 2. The same tendency between HCC patients and control patients was observed.

Laboratory data of the HCC patients at the start of follow-up are shown in Table 3. Patients diagnosed with HCC at a more advanced age had lower baseline serum ALT and AFP levels ($P < 0.0001$ and $P = 0.0043$, respectively) and higher baseline platelet counts ($P = 0.0032$). In Table 4, the oldest group of control patients had lower baseline serum ALT and AFP levels ($P < 0.0001$ and $P = 0.0261$, respectively); however, no significant differences in baseline platelet count were observed.

The results of the Cox proportional hazards model and forward selection method to test factors associated with the age-related development of HCC to patient age at the start of follow-up are shown in Table 5. Ten covariates including age, sex, cirrhosis, alcohol consumption, diabetes mellitus, effect of prior interferon therapy, platelet count, baseline AFP, average integration value of ALT, and the annual rate of platelet count decline were studied. Age, cirrhosis, average integration value of ALT, platelet count, and AFP were significantly associated with hepatocarcinogenesis. However, only cirrhosis and average integration value of ALT were selected as factors significantly associated with hepatocarcinogenesis in patients ≧ 65 or 70 years old. Platelet count was not a significant factor.

Table 1 Profile of HCV-infected HCC patients at the start of follow-up

	Group A ($n = 36$)	Group B ($n = 115$)	Group C ($n = 143$)	Group D ($n = 29$)	P
Sex (female/male)	5/31	43/72	63/80	15/14	0.0016
Age at the start of follow-up† (years)	49 (36–57)	59 (47–66)	66 (52–75)	74 (64–80)	< 0.0001
Duration of observation period until HCC diagnosis† (years)	6.4 (3.1–16.7)	6.9 (3.0–15.8)	8.0 (3.0–17.7)	9.3 (3.0–15.7)	0.0003
Alcohol consumption (≧ 50 g per day/< 50 g per day)	9/27	24/91	26/117	2/27	0.0873
History of blood transfusion (present/absent)	6/30	26/89	35/108	2/27	0.8247
Diabetes mellitus (present/absent)	24/12	40/75	51/92	5/24	0.0008
Prior interferon therapy (SVR/non-SVR/absent)	3/17/16	12/32/71	0/15/128	0/1/28	< 0.0001

†Expressed as median (range).
Group A, diagnosis of HCC at age ≦ 60 years; Group B, 61–70 years; Group C, 71–80 years; Group D, > 80 years.
HCC, hepatocellular carcinoma; HCV, hepatitis C virus; SVR, sustained virologic response.

Table 2 Profile of control patients with HCV infection at the start of follow-up

	Group A' ($n = 30$)	Group B' ($n = 114$)	Group C' ($n = 136$)	Group D' ($n = 43$)	P
Sex (female/male)	7/23	48/66	56/80	20/23	0.1175
Age at the start of follow-up† (years)	48 (40–56)	58 (48–67)	66 (54–75)	74 (65–82)	< 0.0001
Duration of observation period until the end of follow-up† (years)	7.0 (3.0–15.5)	7.8 (3.0–18.7)	8.5 (3.0–17.7)	8.5 (3.6–19.1)	0.0064
Alcohol consumption (≧ 50 g per day / < 50 g per day)	8/22	27/87	20/116	3/40	0.0630
History of blood transfusion (present/absent)	5/25	29/85	40/96	2/41	0.1939
Diabetes mellitus (present/absent)	7/23	38/76	47/89	12/31	0.0758
Prior interferon therapy (SVR/non-SVR/absent)	4/15/11	8/34/72	3/20/113	0/1/42	< 0.0001

†Expressed as median (range).
Group A', age ≦ 60 years at the end of follow-up; Group B', 61–70 years; Group C', 71–80 years; Group D', > 80 years.
HCV, hepatitis C virus; SVR, sustained virologic response.

Clinical features at the time of HCC diagnosis. Platelet counts at the time of HCC diagnosis in groups A, B, C, and group D were 72×10^3/mm^3 (40–192), 84×10^3/mm^3 (28–256), 99×10^3/mm^3 (31–355), and 119×10^3/mm^3 (58–232), respectively. There is a statistically significant trend toward higher platelet counts as the age at HCC diagnosis increases ($P < 0.0001$). In contrast, platelet counts at the end of follow-up in groups A', B', C', and D' were 194×10^3/mm^3 (44–543), 172×10^3/mm^3 (40–484), 177×10^3/mm^3 (21–415), and 193×10^3/mm^3 (52–429), respectively. There is no significant difference between the four groups of control patients ($P = 0.4772$). The annual rate of decline in platelet count, calculated as [platelet count at the start of the study period—platelet count at the time of HCC diagnosis]/duration of the observation period until the diagnosis of HCC, decreased significantly as the age at HCC diagnosis increased, and the annual rate of decline in platelet count, calculated as [platelet count at the start of study period—platelet count at the end of follow-up]/duration of observation period until the end of follow-up in control patients, did not increase significantly as the age at the end of follow-up increased (Fig. 1, $P = 0.0247$ and 0.1571, respectively). The annual rate of platelet count decline was

Table 3 Baseline laboratory data of HCV-infected HCC patients

	Group A ($n = 36$)	Group B ($n = 115$)	Group C ($n = 143$)	Group D ($n = 29$)	P
Platelet count[1] (x 10^3/mm^3)	104 (34–249)	114 (29–253)	125 (44–307)	124 (70–201)	0.0032
Prothrombin time[1] (%)	87 (52–129)	88 (24–119)	85 (22–128)	86 (45–129)	0.6062
Total bilirubin[1] (mg/dL)	0.8 (0.3–1.8)	0.7 (0.2–4.7)	0.7 (0.3–6.7)	0.6 (0.2–1.3)	0.4583
ALT[1] (IU/L)	125 (24–361)	76 (18–387)	64 (8–154)	44 (17–221)	< 0.0001
Child-Pugh classification[1,7] (A or B/C)	33/3	103/12	130/13	24/5	0.5512
HCV genotype[1] (1/2)	26/6	66/24	75/29	15/6	0.4083
HCV viral concentration[1] (log copies/mL)	5.7 (2.7–8.0)	5.0 (2.0–8.0)	5.4 (2.0–6.9)	5.5 (3.0–7.0)	0.4952
AFP[1] (ng/mL)	13.5 (1.8–163.4)	8.4 (1.9–583.4)	7.2 (1.0–372.3)	4.8 (1.2–141.5)	0.0043
AFP-L3[1] (%)	0 (0–56.3)	0 (0–43.6)	0 (0–15.2)	0 (0–7.0)	1.0000
DCP[1] (mAU/mL)	19 (10–154)	19 (10–367)	17 (10–745)	15 (10–182)	0.0958
Cirrhosis (present/absent)	31/5	95/20	112/31	21/8	0.0903

[1]Expressed as median (range).
[2]Data were unavailable for 76 patients.
AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin–reactive fraction of AFP; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin; Group A, diagnosis of HCC at age ≤ 60 years; Group B, 61–70 years; Group C, 71–80 years; Group D, > 80 years; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

Table 4 Baseline laboratory data of control patients with HCV infection

	Group A' ($n = 30$)	Group B' ($n = 114$)	Group C' ($n = 136$)	Group D' ($n = 43$)	P
Platelet count[1] (x 10^3/mm^3)	204 (58–375)	180 (40–540)	187 (51–484)	196 (52–418)	0.4301
Prothrombin time[1] (%)	100 (52–138)	96 (38–153)	96 (48–144)	95 (47–145)	0.3435
Total bilirubin[1] (mg/dL)	0.5 (0.2–1.2)	0.4 (0.2–5.3)	0.4 (0.2–5.3)	0.3 (0.2–1.5)	0.6298
ALT[1] (IU/L)	53 (12–131)	46 (5–490)	35 (8–484)	22 (2–199)	< 0.0001
Child-Pugh classification[1,7] (A or B/C)	30/0	103/11	128/8	40/3	0.1088
HCV genotype[1] (1/2)	15/10	60/23	66/25	12/5	0.0869
HCV viral concentration[2] (log copies/mL)	5.9 (2.7–6.6)	5.7 (2.7–7.3)	5.8 (2.0–7.0)	5.1 (3.0–6.6)	0.1130
AFP[1] (ng/mL)	4.3 (0.8–156.3)	3.1 (0.8–170.3)	3.1 (0.8–219.2)	2.0 (0.8–29.2)	0.0261
AFP-L3[1] (%)	0 (0–26.9)	0 (0–34.2)	0 (0–41.4)	0 (0–5.2)	1.0000
DCP[1] (mAU/mL)	22 (10–122)	19 (10–487)	19 (10–503)	16 (10–30)	0.2549
Cirrhosis (present/absent)	5/25	35/79	48/88	11/32	0.1201

[1]expressed as median (range).
[2]Data were unavailable for 107 patients.
AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin–reactive fraction of AFP; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin; Group A', age ≤ 60 years at the end of follow-up; Group B', 61–70 years; Group C', 71–80 years; Group D', > 80 years; HCV, hepatitis C virus.

Table 5 Factors associated with the development of HCC according to the age at start of follow-up in multivariate analysis

		All patients ($n = 646$) hazard ratio (95% CI)	≥ 60 years ($n = 428$) hazard ratio (95% CI)	≥ 65 years ($n = 255$) hazard ratio (95% CI)	≥ 70 years ($n = 92$) hazard ratio (95% CI)
Age (years)	≤ 60	1			
	> 60, ≤ 70	1.600 (1.240–2.064)			
	> 70	2.738 (1.858–4.036)			
Cirrhosis	Absent	1	1	1	1
	Present	2.165 (1.575–2.978)	2.269 (1.554–3.311)	2.734 (1.724–4.336)	2.962 (1.200–7.310)
Average integration value of ALT (IU/L)	≤ 20	1	1	1	1
	> 20, ≤ 40	4.239 (1.336–13.800)	4.885 (1.179–20.249)	5.243 (1.253–22.020)	12.162 (1.549–95.496)
	> 40, ≤ 60	5.518 (1.725–17.648)	6.661 (1.619–23.397)	6.739 (1.610–28.250)	6.797 (0.854–54.080)
	> 60, ≤ 80	7.182 (2.230–23.130)	9.362 (2.268–38.641)	12.265 (2.867–56.471)	11.183 (1.400–89.317)
	> 80	10.211 (3.175–33.031)	12.249 (2.494–50.894)	13.097 (2.962–57.815)	11.052 (0.964–126.671)
Platelet count (x 10^3/mm^3)	≥ 150	1	1		
	< 150	1.644 (1.237–2.186)	1.728 (1.240–2.409)		
AFP* (ng/mL)	≤ 10	1			
	> 10, ≤ 20	1.406 (1.002–1.971)			
	> 20	1.609 (1.214–2.132)			

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma.

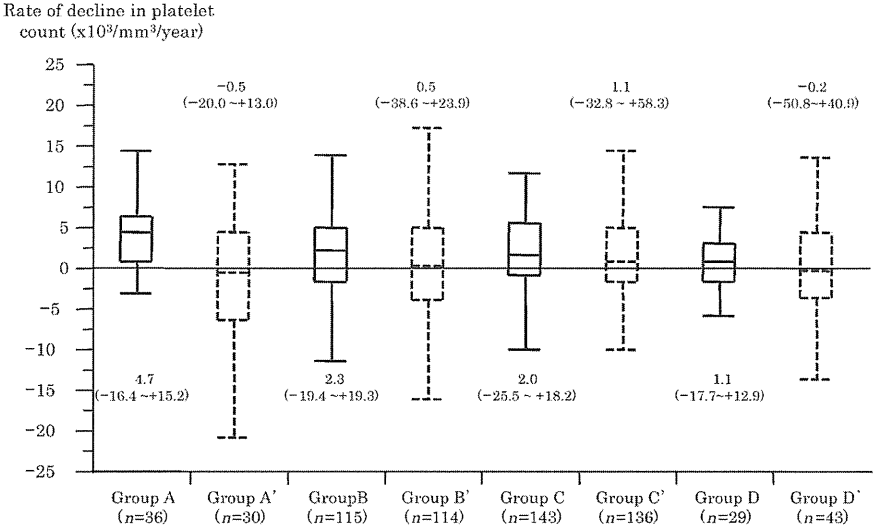

Figure 1 Rate of decline in platelet count prior to hepatocellular carcinoma (HCC) diagnosis in HCC patients and prior to the end of follow-up in control patients. The annual rate of platelet count decline in the period prior to HCC diagnosis was lower in the groups that were older at the time of HCC diagnosis. In control patients, there was no trend toward higher annual rates of platelet count decline in the period prior to the end of follow-up when the patients were classified by age ($P = 0.0247$ and 0.1571, respectively, Jonckheere-Terpstra Test). Group A, HCC diagnosed at age ≤ 60 years; group B, 61–70 years; group C, 71–80 years; group D, > 80 years. group A', control patients ≤ 60 years old at the end of follow-up; group B', 61–70 years; group C', 71–80 years; group D', > 80 years. The annual rate of platelet count decline was significantly lower in group A' than in group A ($P = 0.0039$); however, there were no significant differences when HCC patients in other age groups were compared to their respective matched controls.

lower in group A' than in group A ($P = 0.0039$), and there were no significant differences between group B and group B', group C and group C', and group D and group D'.

The average integration value of ALT in groups A, B, C, and D was 80.9 IU/L (25.3–179.3), 62.3 IU/L (14.5–167.9), 59.0 IU/L (9.9–134.1), and 44.9 IU/L (22.7–91.9), respectively. The average integration value of ALT was significantly lower in patients diagnosed with HCC at an older age (Fig. 2, $P < 0.0001$). There was a similar trend among control patients (Fig. 2, $P < 0.0001$). The average integration values of ALT in groups A', B', C', and D' were significantly lower than in groups A, B, C, and D, respectively ($P < 0.0001$).

Patient profiles at the time of HCC diagnosis are shown in Table 6. There were no significant differences in tumor characteristics and levels of tumor markers among the age groups. Fewer patients in Group D underwent hepatic resection ($P = 0.0293$).

Survival rates according to age at HCC diagnosis. Five and 10-year cumulative survival rates of groups A, B, C, and D were 44.2%, 58.2%, 44.3%, and 33.3% and 22.7%, 31.2%, 26.6%, and not available, respectively (Fig. 3). There were no significant differences in the cumulative survival rate among the four groups.

Discussion

In Japan, the average age of patients with chronic hepatitis, cirrhosis, or HCV-associated HCC is increasing. The number of deaths due to these diseases is also increasing. The age-specific prevalence of HCV seropositivity in the USA is about 30 years below that in Japan; thus, a majority of patients in the USA with chronic HCV infection will reach an advanced age in the near future.[5]

In our study, elderly HCC patients have high platelet counts and low ALT values. In addition, multivariate analysis using propensity-matched control patients revealed that the presence of cirrhosis and high ALT levels (> 20 IU/L) are significantly associated with the development of HCC. However, platelet count is not significantly associated with hepatocarcinogenesis in elderly HCV carriers (≥ 65 years). Physicians should be aware that patients aged 65 years or older could develop HCC regardless of their platelet count.

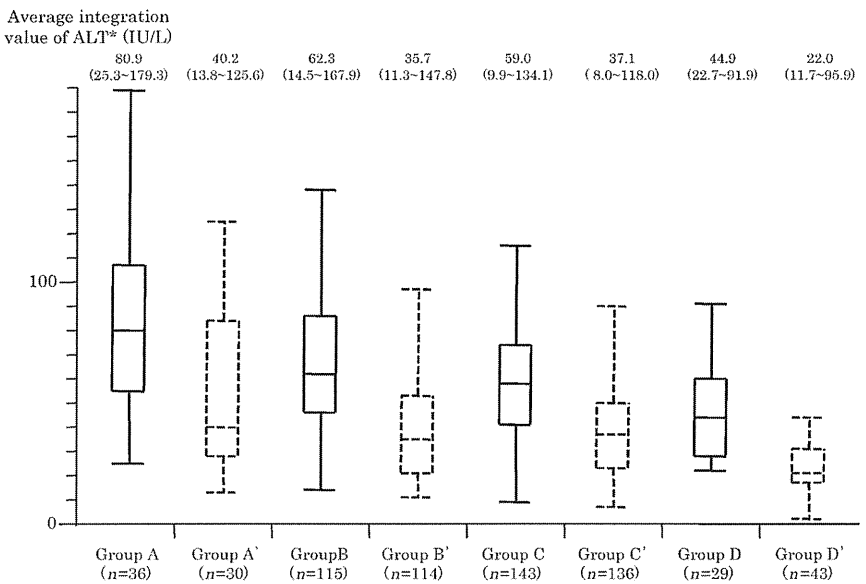

Figure 2 Average integration values of alanine aminotransferase (ALT) prior to HCC diagnosis in HCC patients and prior to the end of follow-up in control patients. Patients who were older at the time of HCC diagnosis had lower average integration values of ALT in the period prior to HCC diagnosis. In control patients, the average integration values of ALT in the period prior to the end of follow-up were lower in the groups that were older at the end of follow-up ($P < 0.0001$ and < 0.0001, respectively, Jonckheere-Terpstra Test). Average integration values of ALT in groups A', B', C', and D' were significantly lower than in groups A, B, C, and D, respectively ($P < 0.0001$).

Table 6 Profile of HCV-infected HCC patients at the time of HCC diagnosis

	Group A ($n = 36$)	Group B ($n = 115$)	Group C ($n = 143$)	Group D ($n = 29$)	*P*
AFP† (ng/mL)	23.9 (0.8–500)	19.8 (0.6–10500)	12.8 (0.8–12680)	17.8 (0.8–99720)	0.2347
AFP-L3† (%)	0 (0–89)	0 (0–87.2)	0 (0–81.0)	0 (0–40.7)	1.0000
DCP† (mAU/mL)	36 (10–36164)	35 (10–5941)	32 (10–50904)	24 (10–6229)	0.5650
Tumor size† (cm)	2.0 (0.8–10.0)	2.0 (0.3–8.8)	2.0 (0.6–11.4)	2.3 (1.0–9.0)	0.3754
Number of tumors†	1 (1–6)	1 (1–8)	1 (1–10)	1 (1–4)	1.0000
Portal thrombus (present/absent)	2/34	3/112	6/137	0/29	0.3293
Stage (I/2/3/4)	14/15/5/2	41/53/21/0	50/61/29/3	10/12/7/0	0.4957
Initial treatment (HR/PT/TACE/none)	9/18/4/5	47/44/16/8	51/47/33/12	4/11/9/5	0.0293

†Expressed as median (range).
AFP, α-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive fraction of AFP; DCP, des-γ-carboxy prothrombin; Group A, diagnosis of HCC at age ≤ 60 years; Group B, 61–70 years; Group C, 71–80 years; Group D, > 80 years; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hepatic resection; PT; percutaneous treatment including ethanol injection therapy, microwave coagulation therapy, and radiofrequency ablation therapy; TACE, transcatheter arterial chemoembolization.

The male-to-female ratio of HCC patients in Japan has decreased from 4.5 in 1984–1985 to 2.5 in 2002–2003.[1] It is well known that the mean age of female HCC patients with HCV infection is higher than that of males.[18,19] The increased proportion of female patients is considered a result of more older patients with HCV-related HCC. In our study, the proportion of female patients was the highest in group D. Further investigation of the role of sex in hepatocarcinogenesis is needed.

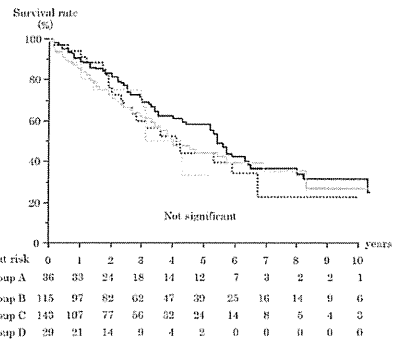

Figure 3 Cumulative survival rate of groups A, B, C, and D according to age at hepatocellular carcinoma (HCC) diagnosis. Kaplan-Meier curves showing the survival rate stratified by age at HCC diagnosis. There were no significant differences in the survival rate among the four groups. ——, A group (≤ 60 years, $n = 36$); ·····, B group (61–70 years, $n = 115$); , C group (71–80 years, $n = 143$); , D group (> 80 years, $n = 29$).

We previously reported that the average integration value of ALT was associated with the cumulative incidence of hepatocarcinogenesis and that minimizing ALT is necessary for the prevention of hepatocarcinogenesis.[20] In addition, we demonstrated a 6.242-fold higher (95% confidence interval: 1.499–25.987) cumulative incidence of hepatocarcinogenesis in patients with average ALT integration values between 20 and 40 IU/L (within the current normal range) than in patients with 20 IU/L or below.[21] In this study, the average integration value of ALT significantly decreased as the age at HCC diagnosis increased. Especially in group D, the average integration value of ALT was 44.9 IU/L (range, 22.7–91.9 IU/L), which is near the upper limit of the conventional reference range of ALT (40 IU/L). There was the same tendency in control patients; however, average integration values of ALT were lower in control patients than HCC patients in each corresponding age group. These data suggest close surveillance for HCC is important even if older patients (≥ 65 years) have low ALT values.

It is likely that low platelet counts account for a large proportion of patients with cirrhosis, consistent with the theory that HCC develops in patients with progressive or advanced liver disease. Cirrhosis is an established risk factor for HCC in patients with HCV.[22,23] It is generally accepted that platelet count is a surrogate marker of liver fibrosis.[24,25] Platelet counts were highest in group D, both at the start of follow-up and at the time of HCC diagnosis. In contrast, there were no differences in platelet counts among control patients without HCC. It is particularly worth noting that group D had the smallest annual decline in platelet count, at levels comparable to the control patients. A previous report showed that the rate of progression of fibrosis to cirrhosis was accelerated by aging.[24] The precise mechanism of this discrepancy is uncertain. Probably, differences in patient selection might account for this discrepancy. We hypothesize that in our study, the increased rate of annual decline in platelet count may be linked to accelerated carcinogenesis occurring in the younger patients. Group D also had the lowest values of AFP, which is considered a marker of hepatic regeneration as well as a HCC tumor marker in viral hepatitis.[26] Taken together, this suggests a weaker inflammatory response in older patients. Further investigation is necessary.

Why do elderly patients progress to HCC even though liver function appears stable? Aging is associated with a number of events at the molecular, cellular, and physiological level that influence carcinogenesis and subsequent cancer growth.[22] Age may be considered as a progressive loss of stress tolerance due to declines in the functional reserve of multiple organ systems.[27] It has been hypothesized that age-associated declines in DNA repair[28] contribute to the development of HCC. The precise relationship between aging and hepatocarcinogenesis remains uncertain. Further assessment of the role of aging in the progression of HCV is needed.

We found no difference in tumor stage among the four groups. The younger groups A and B tended to receive curative therapy more often than the older groups C and D. However, there were no significant differences in survival. We hypothesize that this is due to the aggressive multiple treatments received by elderly patients with good liver function.

One limitation of our study is that histological confirmation was available in only 234 patients (36.2%). However, it is not practical to perform biopsies on all patients because of potential complications. Lu *et al.* reported that the best cutoff platelet count for the diagnosis of cirrhosis is 150×10^3 /mm^3.[29] Therefore, we employed platelet count as a surrogate marker of liver fibrosis in this study.

In conclusion, we demonstrated that elderly HCV-positive patients (≥ 65 years old) with low ALT values developed HCC regardless of their platelet counts. This finding should be taken into account when designating the most suitable HCC surveillance protocol. The optimal screening interval for HCV-infected patients aged 65 years older should be three to four months like cirrhotic patients even in the absence of cirrhosis.

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Research Article

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Effect of nucleos(t)ide analogue therapy on hepatocarcinogenesis in chronic hepatitis B patients: A propensity score analysis

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Background & Aims: Some patients with chronic hepatitis B virus (HBV) infection progress to hepatocellular carcinoma (HCC). However, the long-term effect of nucleos(t)ide analogue (NA) therapy on progression to HCC is unclear.
Methods: Therefore, we compared chronic hepatitis B patients who received NA therapy to those who did not, using a propensity analysis.
Results: Of 785 consecutive HBV carriers between 1998 and 2008, 117 patients who received NA therapy and 117 patients who did not, were selected by eligibility criteria and propensity score matching. Factors associated with the development of HCC were analyzed. In the follow-up period, HCC developed in 57 of 234 patients (24.4%). Factors significantly associated with the incidence of HCC, as determined by Cox proportional hazards models, include higher age (hazard ratio, 4.36 [95% confidence interval, 1.33–14.29], $p = 0.015$), NA treatment (0.28 [0.13–0.62], $p = 0.002$), basal core promoter (BCP) mutations (12.74 [1.74–93.11], $p = 0.012$), high HBV core-related antigen (HBcrAg) (2.77 [1.07–7.17], $p = 0.036$), and high gamma glutamyl transpeptidase levels (2.76 [1.49–5.12], $p = 0.001$).
Conclusions: NA therapy reduced the risk of HCC compared with untreated controls. Higher serum levels of HBcrAg and BCP mutations are associated with progression to HCC, independent of NA therapy.

Introduction

An estimated 350 million individuals worldwide are chronically infected with hepatitis B virus (HBV), of whom 1 million die annually from HBV-related liver disease [1]. Chronic HBV infection is recognized as a major risk factor for the development of hepatocellular carcinoma (HCC) [1,2]. Hepatitis B surface antigen (HBsAg)-positive patients have a 70-fold increased risk of developing HCC compared to HBsAg seronegative counterparts [3,4]. HBV infection is endemic in Southeast Asia, China, Taiwan, Korea, and sub-Saharan Africa, where up to 85–95% of patients with HCC are HBsAg positive [5]. HCC is the third and fifth leading cause of cancer death in men and women, respectively, and the number of deaths and the mortality rate from HCC have greatly increased in Japan since 1975 [6]. Hepatitis C virus (HCV)-related HCC accounts for 75% of all HCCs in Japan and HBV-related HCC accounts for 15% [6].

In 2004, Liaw *et al.* reported a significant reduction in HCC in 651 adults receiving lamivudine after adjustment for baseline variables (hazard ratio, 0.49 [95% confidence interval (95% CI), 0.25–0.99], $p = 0.047$) [7]. However, the results were not significant after exclusion of 5 patients who developed HCC within 1 year of randomization (0.47 [0.22–1.00], $p = 0.052$). Therefore, in 2009, the National Institutes of Health Consensus Development Conference concluded that there was insufficient evidence to assess whether nucleos(t)ide analogue (NA) therapy can prevent the development of HCC [8].

The long-term use of lamivudine has not been recommended because of tyrosine–methionine–aspartate–aspartate (YMDD) mutations, which have occasionally been associated with severe and even fatal flares of hepatitis [9,10]. Therefore, adefovir dipivoxil should be added immediately in patients with virological or biochemical breakthroughs or no response. Currently, there are 2 nucleoside agents (lamivudine, entecavir) and 1 nucleotide agent (adefovir dipivoxil) available for treatment of HBV infection in Japan. The agent with the higher genetic barrier to resistance, entecavir, is considered the initial drug of choice [11]. Recently, 3 studies on lamivudine suggested that long-term sustained viral suppression was associated with a reduced likelihood of developing HCC [12–14].

In this study, we sought to determine if NA therapy was associated with a reduction in the development of HCC. Since the validity of treatment effects in observational studies may be limited by selection bias and confounding factors, we performed a propensity analysis [15].

Keywords: HBcrAg; BCP; Gamma-GTP; Average integration value; HBV DNA.
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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; NA, nucleos(t)ide analogue; HBcrAg, HBV core-related antigen; BCP, basal core promoter; gamma-GTP, gamma glutamyl transpeptidase.

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