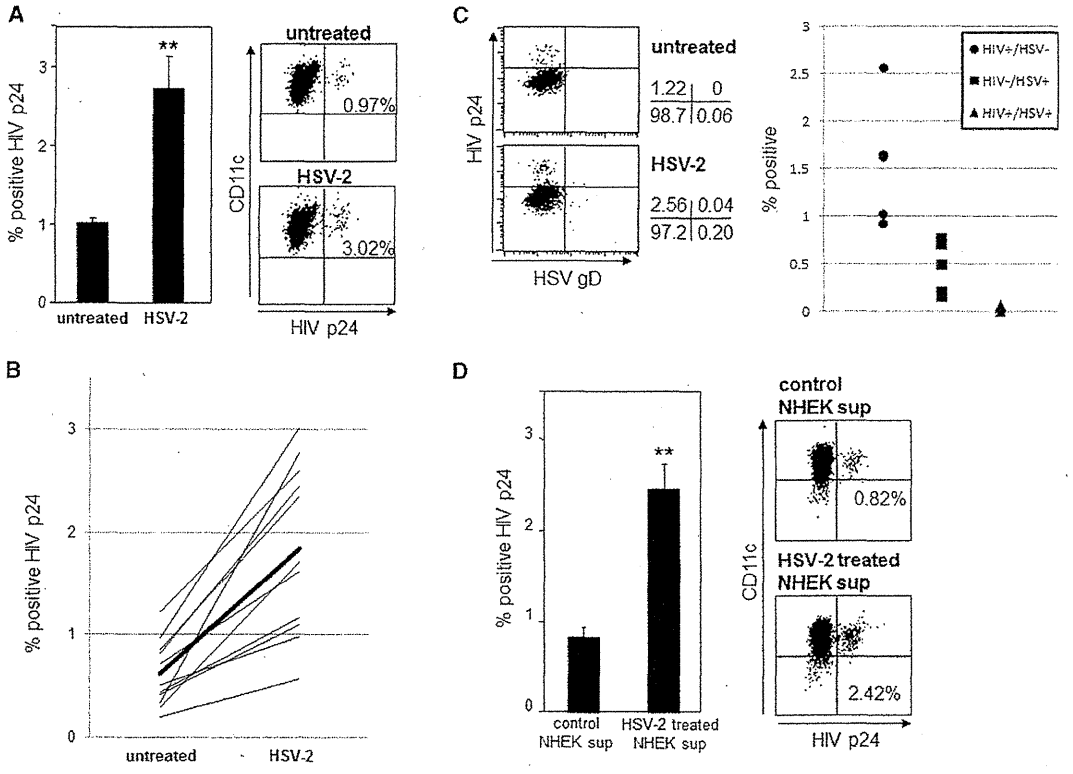

Figure 1. HSV-2-Infected Epithelial Cells Augment HIV Infection in LCs

(A–C) Epithelial sheets were preincubated with HSV-2 and then exposed to R5 HIV. Epithelial sheets were floated on culture medium to allow migration of LCs from the explants. Emigrating cells from the epidermal sheets were collected 3 days following HIV exposure. HIV-infected LCs were assessed by HIV p24 intracellular staining in langerin$^+$ CD11c$^+$ LCs (A) and the results of 11 separate experiments with different donors are summarized (B). HSV– and/or HIV-infected LCs were assessed by HIV p24 and HSV gD intracellular staining in CD11c$^+$ LCs, and the results from five different donors are summarized (C).

(D) mLCs were preincubated with the supernatants from NHEKs treated with or without HSV-2, and then exposed to R5 HIV. HIV p24$^+$ cells were assessed in langerin$^+$ CD11c$^+$ mLCs at day 7.

(A, C, and D) Representative flow cytometric analyses are shown. (A and D) Results are shown as means ± SD (n = 3) (**p < 0.01). See also Figure S1.

90% of SIV-infected cells were found to be LCs (Hu et al., 2000). Because only a single layer of columnar epithelium guards the endocervix and the transformation zone, the mucosal barrier can be easily breached by mechanisms such as the microtrauma associated with sexual intercourse, which provides immediate access to target cells, especially CD4$^+$ T cells, in the submucosa (Haase, 2010). Indeed, it has been shown that following mucosal exposure to high doses of SIV, virus can gain access through breaks in the mucosal epithelial barrier and infect resting CD4$^+$ T cells in the submucosa (Haase, 2010). Since molecules targeting CCR5 completely protected against mucosal transmission of SHIV (Lederman et al., 2004), CD4/CCR5-mediated de novo infection of LCs and/or CD4$^+$ T cells is considered to be a major pathway involved in sexual transmission of HIV.

Several mechanisms have been proposed to explain enhanced sexual transmission of HIV during active STD infection, including breakdown of epithelial barriers (i.e., ulceration) with direct inoculation of HIV into the blood (Cunningham et al., 1985), presence of inflammatory leukocytes that act as targets (Zhu et al., 2009), and coinfection of cells by HIV and STD pathogens. Biological mechanisms responsible for greater HIV transmission rates in the presence of genital herpes infections, however, are as of yet unknown. Recently, we and others have suggested that HIV susceptibility of LCs could be directly enhanced by pathogens and indirectly enhanced by inflammatory factors during STD, thereby leading to more likely sexual transmission of HIV (de Jong et al., 2008; Ogawa et al., 2009). In this report, we found that HSV-2 primarily infected epithelial cells and then markedly enhanced HIV infection in adjacent LCs. Interestingly, mechanistic studies revealed that LL-37 produced by HSV-2-infected epithelial cells upregulated CD4 and CCR5 on the surface of bystander LCs, thereby enhancing HIV infection in these cells. These findings may lead to new strategies designed to block sexual transmission of HIV.

RESULTS

HSV-2 Indirectly Enhances HIV Susceptibility in LCs via Interaction with Epithelial Cells

We first examined whether HSV-2 modulates HIV susceptibility of LCs by using an ex vivo skin explant model, whereby resident LCs within epithelial tissue are exposed to HIV and then allowed to emigrate from tissue, thus mimicking conditions that occur following mucosal exposure to HIV (Kawamura et al., 2000).

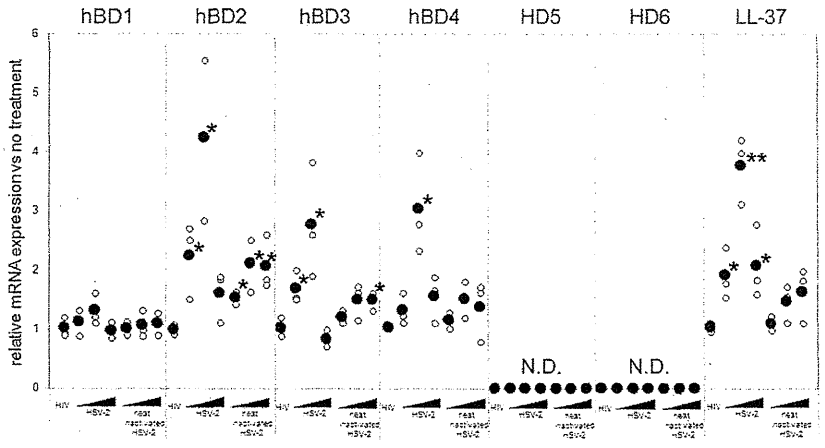

Figure 2. HSV-2 Increases Expression of Human β Defensin-2, Defensin-3, Defensin-4, and LL-37 in Normal Human Epithelial Cells

HIV-1 (10^5 $TCID_{50}$), HSV-2 ($1 \times 10^4 - 1 \times 10^6$ PFU), and heat-inactivated HSV-2 ($1 \times 10^4 - 1 \times 10^6$ PFU) were exposed to NHEKs for 1 hr and then washed twice. Cells were incubated in culture medium for 12 hr, and the mRNA expression of indicated AMPs was determined by qPCR. Results are shown as mean relative mRNA expressions (○) from three different experiments and mean the average (●) of those (*$p < 0.05$; **$p < 0.01$). See also Figure S2.

Epidermal sheets obtained from suction blister roofs were exposed to HSV-2 strain G at 1×10^6 PFU/tissue for 1 hr, and then exposed to R5-tropic HIV-1_{BaL} for 2 hr. Three days later, to quantify numbers of HIV-infected LCs at the single-cell level, cells emigrating from the explants were stained with anti-CD11c, anti-langerin, and anti-HIV p24 mAbs. Intracellular staining for HIV p24 represents productive HIV replication within LCs, since expression can be completely blocked by AZT (Kawamura et al., 2003). The numbers of LCs emigrating from individual explants were determined, and the mean yield ± SD was HSV−/HIV−; 1.04 ± 0.25 × 10^4, HSV−/HIV+; 1.13 ± 0.31 × 10^4, HSV+/HIV−; 1.25 ± 0.27 × 10^4; and HSV+/HIV+; 1.21 ± 0.25 × 10^4 (n = 3). Thus, the number of LCs recovered from the skin explants was not significantly affected by HSV-2 or HIV exposure. However, preincubation of epithelial sheets with HSV-2 significantly increased the percentage of HIV $p24^+$ cells within $langerin^+$ $CD11c^+$ LCs approximately 3-fold as compared to LCs emigrating from nonexposed epithelial sheets (Figure 1A). The results of 11 separate experiments with different skin donors are summarized in Figure 1 B (mean percentage HIV $p24^+$ LCs ± SD = 0.61 ± 0.31 with no HSV-2; 1.85 ± 0.79 with HSV-2 preincubation, p = 0.0002, n = 11). To assess the ratio of individual HSV-2- and/or HIV-infected LCs emigrating from explants, cells were collected from cultures and stained with anti-CD11c, anti-HSV gD, and anti-HIV p24 mAbs. A recent study in mice showed that HSV impeded emigration of infected LCs by inducing apoptosis and by blocking E-cadherin downregulation (Miller et al., 2011b). Consistent with this finding, the percentage of HSV-2-infected emigrating LCs was much less when compared with LCs that remained within explants at day 3 (Figure 1C and see Figure S1 online). More importantly, we found that HSV-2/HIV-coinfected emigrating LCs were rarely detected (mean percentage of HIV-1 $p24^+$/HSV-2 gD^+ LCs ± SD = 0.04 ± 0.02, n = 5, Figure 1C), suggesting that HSV may not directly modulate HIV susceptibility in LCs. Indeed, supernatants from epithelial cell cultures of normal human epidermal keratinocytes (NHEKs) treated with HSV-2 also increased the percentage of HIV p24+ monocyte-derived LCs (mLCs), even though mLCs were not exposed to HSV-2 (Figure 1D). Of note, the magnitude of HIV susceptibility in mLCs enhanced by HSV-treated NHEK supernatants was comparable to that in emigrating LCs in the epithelial explant experiments. Enhancement of HIV infection by supernatants from HSV-2-treated NHEKs was dependent on the dose of HSV-2, and supernatants from heat-inactivated HSV-2-treated NHEKs did not affect HIV susceptibility in mLCs (Figure S2). HIV susceptibility in mLCs was also enhanced when we infected NHEKs with a second HSV-2 strain, 186 (data not shown). Taken together, these results suggest that HSV-2 indirectly mediates HIV infection of epidermal LCs by a soluble factor or factors released by HSV-2-infected epithelial cells.

HSV-2 Augments the Production of Antimicrobial Peptides from Keratinocytes, and LL-37 Enhances HIV Infection in LCs

In STDs, antimicrobial peptides (AMPs), including defensins and cathelicidin, are the key effector molecules of mucosal innate and adaptive immunity. Human vaginal epithelial cells and epidermal keratinocytes can produce human α defensin-5 (HD5), HD6 and human β defensin-1 (hBD1), hBD2, hBD3, hBD4, and the sole cathelicidin in humans, LL-37. Certain defensins (e.g., hBD1) are expressed constitutively, and others (e.g., hBD2 and hBD3) show increased expression in response to inflammation or infection (Klotman and Chang, 2006). Several reports have indicated that several of these AMPs modulate HIV infectivity in peripheral blood mononuclear cells (PBMC) or in $CD4^+$ T cells (Bergman et al., 2007; Klotman et al., 2008; Quiñones-Mateu et al., 2003; Sun et al., 2005). Thus, we investigated whether HSV-2 induced AMPs production in keratinocytes. NHEKs were incubated with HSV-2 ($1 \times 10^4 - 1 \times 10^6$ PFU) or HIV-1 (1×10^5 $TCID_{50}$), and relative mRNA expression levels of AMPs were determined by quantitative RT-PCR. Interestingly, HSV-2 significantly increased the expression of hBD2, hBD3, hBD4, and LL-37, whereas neither HIV nor heat-inactivated HSV-2 affected expression of hBD3, hBD4, and LL-37 (Figure 2).

To determine whether keratinocyte-derived AMPs affect HIV susceptibility of LCs, mLCs were stimulated with AMPs or TNF-α, as a positive control, for 24 hr before exposure to HIV-1. Strikingly, only LL-37 significantly increased the percentage of HIV $p24^+$ mLCs (Figure 3A). This infection-enhancing effect of LL-37 was observed in a dose-dependent manner, utilizing concentrations of LL-37 observed in physiologic conditions (Ong et al., 2002; Yamasaki et al., 2007) (Figure 3B). Interestingly, LL-37 also significantly upregulated surface expression

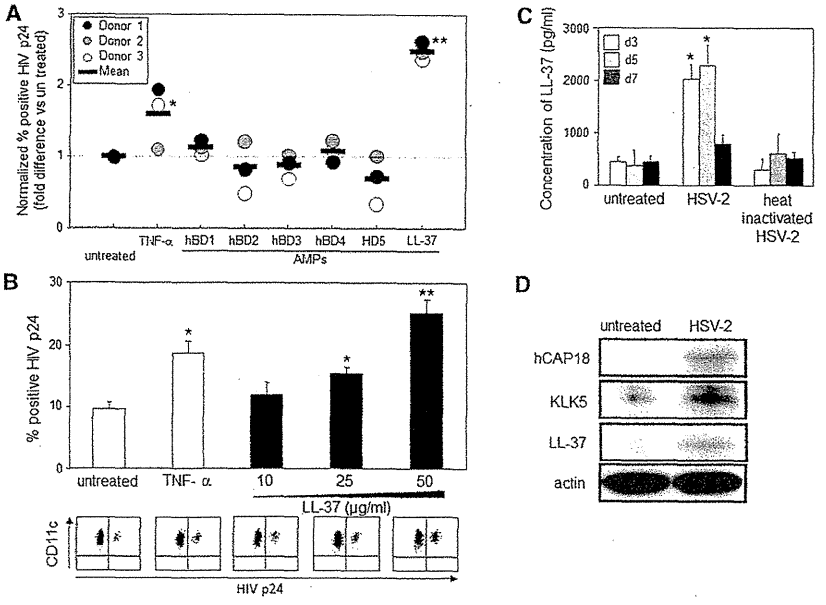

Figure 3. LL-37 Enhances HIV Susceptibility in mLCs

mLCs were stimulated with the indicated AMPs or rhTNF-α 24 hr prior to HIV exposure. To assess HIV infection levels, mLCs were collected 7 days after HIV exposure, and HIV p24$^+$ cells were quantified in langerin$^+$ CD11c$^+$ mLCs.

(A) Each circle indicates the normalized percentage of positive cells for HIV p24; mean values obtained from different donors are shown as horizontal marks.

(B) The percentage of positive cells for HIV p24 in langerin$^+$ CD11c$^+$ mLCs and representative flow cytometric analyses following LL-37 stimulation.

(C) NHEKs were exposed to HSV-2 or heat-inactivated HSV-2. Following culture for the indicated number of days, LL-37 levels were measured in supernatants by ELISA. Results are shown as means ± SD (n = 3) (*$p < 0.05$).

(D) NHEKs were treated with HSV-2 for 3 days and then lysed. The expression of hCAP18, KLK5, and LL-37 was determined by western blot analysis. All data shown represent at least two separate experiments. See also Figure S3.

of CD86, CD83, and CCR7 on mLCs (Figure S3), indicating that LL-37 induces LC maturation.

To confirm whether HSV-2-treated NHEKs could produce LL-37 protein, we measured LL-37 protein levels in culture supernatants from NHEKs treated with medium alone, HSV-2, or heat-inactivated HSV-2 by ELISA. HSV-2 significantly induced production of LL-37 in NHEKs, which peaked at day 5 (Figure 3C). Expression levels of kallikrein 5 (KLK5) have been shown to parallel induction of LL-37 in KCs, since the activity of cathelicidin is controlled by enzymatic processing of the proform hCAP18 to a mature peptide LL-37 by KLK5, a serine protease (Morizane et al., 2010; Yamasaki et al., 2006). Therefore, we measured protein levels of LL-37, hCAP18, and KLK5 in NHEKs treated with medium alone or HSV-2. As shown in Figure 3D, HSV-2 induced production of LL-37 in NHEKs. Similarly, HSV-2 increased expression of hCAP18 as well as KLK5, and these protein levels coincided with the induction of LL-37 in NHEKs (Figure 3D).

To further confirm the participation of LL-37 in this enhancement, we used RNA interference (siRNA) to block LL-37 production. Protein levels were quantified by western blotting followed by densitometry analysis. Transfection of siRNA targeting LL-37 induced an efficient knockdown in NHEKs (55% downregulation; Figure 4A). In line with the results of western blot analyses, siRNA-mediated interference of LL-37 in NHEKs significantly reduced enhancement of HIV infection in mLCs by supernatants from HSV-2-treated NHEKs, in comparison with control siRNA targeting an irrelevant sequence (Figure 4B). Based on these results, we conclude that enhanced HIV infection in mLCs by supernatants from HSV-2-treated NHEKs is, at least in part, mediated by LL-37.

Recently, TNF-α derived from KCs has also been shown to enhance HIV susceptibility of LCs (de Jong et al., 2008; Ogawa et al., 2009). In our experiments, however, TNF-α was not detected in culture supernatants from NHEKs treated by HSV-2 (data not shown), consistent with a recent report (de Jong et al., 2010). In addition, preincubation of supernatants from HSV-2-treated NHEKs with an anti-TNF-α neutralizing mAb, prior to exposing mLCs, did not affect HIV susceptibility in mLCs (Figure S4).

LL-37 Enhances Surface Expression of CD4 and CCR5 on mLCs

Previous studies have revealed that langerin expressed on LCs is a natural barrier to HIV infection because HIV virions captured by langerin are internalized into LC Birbeck granules and degraded (de Witte et al., 2007). In addition, APOBEC3G (A3G) and SAM domain and HD domain 1 (SAMHD1) has been recently shown to function as a potent postentry cellular restriction factor for HIV in DCs or LCs (Hrecka et al., 2011; Laguette et al., 2011; Ogawa et al., 2009; Pion et al., 2006). Therefore, we next examined whether LL-37 affects the expression levels of these molecules in mLCs. LL-37 stimulation did not affect the expression of langerin, A3G, or SAMHD1 (Figures 4C and 4D and Figure S5). In contrast, LL-37 significantly increased surface expression of CD4 and CCR5 on mLCs (Figure 4C). In addition, siRNA-mediated interference of LL-37 in NHEKs significantly reduced the enhancement of surface expression of CD4 and CCR5 in mLCs by supernatants from HSV-2-treated NHEKs, in comparison with control siRNA targeting an irrelevant sequence (Figure 4C). Thus, our results suggest that LL-37 enhances HIV infection in LCs by increasing surface expression of HIV receptors, rather than by modulating restriction factors such as langerin, A3G, or SAMHD1.

Since LL-37 upregulated surface expression of CD4 and CCR5 in LCs, we next examined whether LL-37 specifically enhanced R5-tropic HIV entry into LCs by using single-round infection assays with pseudotyped viruses containing a luciferase reporter and different envelope proteins (Env): Env from either R5 HIV-1 (JR-FL; R5), X4 HIV-1 (IIIB; X4), or vesicular stomatitis virus (VSV-G). As expected, we found that LL-37 pretreatment enhanced the infectivity of mLCs to R5-VSV in

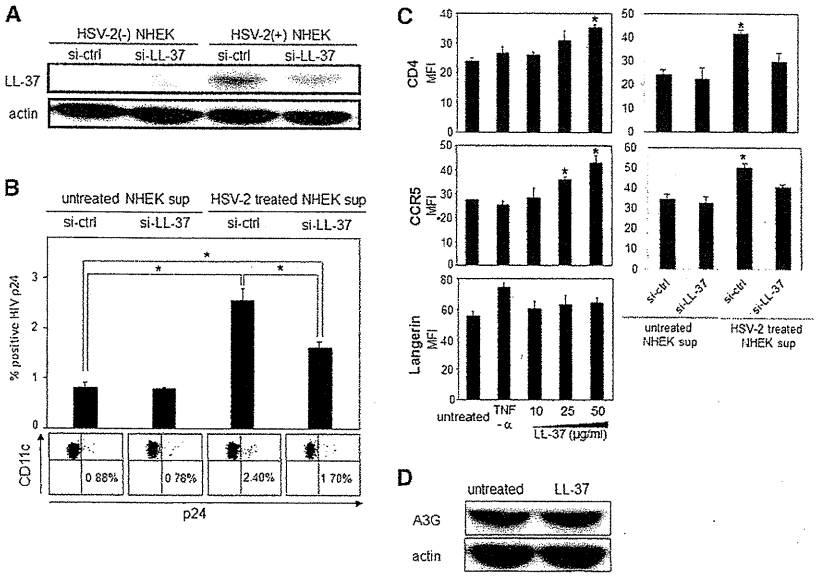

Figure 4. Silencing of LL-37 in HSV-2-Infected NHEKs Abrogates Enhanced HIV Infectivity in mLCs

(A) NHEKs were transfected with control or LL-37 siRNA and then exposed with or without HSV-2. Cells were lysed and then determined the expression of LL-37 by western blot analysis.

(B) mLCs were incubated with indicated culture supernatants for 12 hr and then exposed to R5 HIV. mLCs were collected 7 days after the HIV exposure, and HIV $p24^+$ cells were assessed in $langerin^+$ $CD11c^+$ mLCs. Representative flow cytometric analyses of CD11c and p24 mAb double-stained cells are shown.

(C) mLCs were stimulated with TNF-α or LL-37 at the indicated concentrations or indicated culture supernatants for 24 hr. The expression of CD4, CCR5, and langerin was assessed by flow cytometry.

(D) The expression of A3G was determined by western blot analysis. Results are shown as means ± SD (n = 3) (*$p < 0.05$). All data shown represent at least two separate experiments. See also Figure S4.

a dose-dependent manner, but LL-37 did not affect infection with VSV-G (Figure 5A). Consistent with previous findings (Kawamura et al., 2001), mLCs were resistant to X4-VSV, even after LL-37 treatment. These results provide direct evidence that LL-37, which upregulates surface expression of CD4 and CCR5 in LCs, promotes increased R5 HIV entry into these cells. Furthermore, similar effects of LL-37 were observed when mLCs were infected with R5 HIV primary isolates: JR-FL and AD8 (Figures 5B and 5C).

LL-37 Decreases HIV Infectivity in mDCs

We next examined whether LL-37 affects HIV infectivity in non-LC-like DCs. Similar to mLCs, LL-37 significantly upregulated surface expression of CD86 and CCR7 on monocyte-derived DCs (mDCs, Figure S3), indicating that LL-37 induces DC maturation. In marked contrast to mLCs, however, there was inhibition of HIV infection in mDCs when these cells were preincubated with LL-37 prior to HIV exposure (Figure 6A), indicating that LL-37 effects on HIV infectivity are differentially regulated in LCs and DCs. LL-37 did not affect CD4 or A3G expression in DCs but markedly downregulated surface expression of CCR5 and DC-SIGN (Figures 6B and 6C). It has been shown that DC-SIGN binds HIV and plays a critical role for HIV replication in mDCs (Gringhuis et al., 2010). These results, in contrast to mLCs, suggest that decreased HIV infection levels observed in LL-37-treated mDCs may be due to downregulation of DC-SIGN and/or CCR5 on their cell surfaces. Taken together, our results indicated the presence of exclusive machinery to augment HIV infection by LL-37 in LC in contrast to that in $CD4^+$ T cells (Bergman et al., 2007) and mDCs.

LL-37 Enhances HIV Transmission from LCs to $CD4^+$ T Cells

We next examined whether LL-37 affected HIV transmission from LCs to cocultured $CD4^+$ T cells. mLCs or mDCs were stimulated with AMPs or TNF-α for 24 hr, exposed to $HIV\text{-}1_{Ba\text{-}L}$, and then cocultured with allogeneic $CD4^+$ T cells for 12 days. Consistent with results showing that LL-37 increases HIV infection levels in mLCs (Figure 3A), preincubation of mLCs with LL-37 significantly enhanced subsequent HIV transmission from mLCs to $CD4^+$ T cells in a dose-dependent manner; preincubation with other AMPs did not affect HIV transmission levels in mLC-T cell cocultures (Figure 7A). By contrast, HIV transmission from mDCs to $CD4^+$ T cells was significantly decreased by preincubation of mDCs with LL-37 (Figure 7B), consistent with decreased HIV infection levels in LL-37-treated mDCs (Figure 6A).

DISCUSSION

LCs are generally believed to be one of the cell types that plays a pivotal role in the dissemination of virus during sexual transmission of HIV. To understand the biologic mechanisms by which HSV-2 increases acquisition of HIV, we tested the hypothesis that HSV-2 modulates LC susceptibility to HIV. As expected, we found that HSV-2 enhances HIV susceptibility of LCs within epithelial tissue (Figure 1), consistent with a recent finding that HSV-2 directly enhances HIV susceptibility in LCs (de Jong et al., 2010). However, in our ex vivo explant model, the percentage of HSV/HIV-coinfected LCs was quite low. Instead, our findings suggested that HSV-2 increases HIV susceptibility in LCs by indirect (i.e., epithelial cell-dependent) mechanisms. More specifically, we show here that LL-37 produced by HSV-2-infected epithelial cells enhances HIV infection of LC, most likely by increasing surface expression of CD4 and CCR5 on these cells.

There are conflicting prior reports on how defensins affect HIV infectivity. A variety of anti-HIV activities for hBD2 and hBD3 have been reported, including direct inhibition of virions, indirect inhibition of HIV replication, and downregulation of HIV coreceptors (Klotman and Chang, 2006; Quiñones-Mateu et al., 2003). By contrast, other studies have shown increased HIV infection of

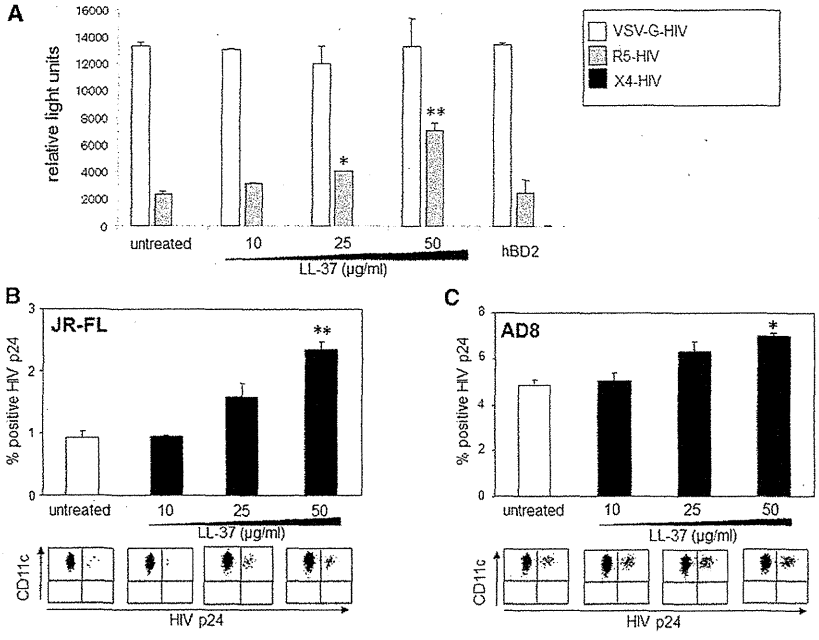

Figure 5. LL-37 Enhances mLCs Susceptibility to R5-HIV and R5 HIV Primary Isolates

mLCs were stimulated with LL-37 at the indicated concentration or hBD2 as control, and then exposed to pseudotyped viruses (R5-HIV, X4-HIV or VSV-G-HIV) for 72 hr (A) or R5 HIV primary isolates (JR-FL or AD8) for 2 hr (B and C). To assess pseudotyped virus infection levels, the average luciferase activity was calculated as relative light units (A). To assess primary HIV infection levels, mLCs were collected 7 days after the HIV exposure, and HIV p24$^+$ cells were assessed in langerin$^+$ CD11c$^+$ mLCs (upper panels, % of positive cells for HIV p24 in langerin$^+$ CD11c$^+$ mLCs; and lower panels, representative flow cytometric analyses following LL-37 stimulation). Results are shown as means ± SD (*$p < 0.05$; **$p < 0.01$). All data shown represent at least two separate experiments. See also Figure S5.

primary CD4$^+$ T cells by HD5 and HD6, and no effects on cell-surface HIV coreceptor expression by hBD1 and hBD2 (Klotman et al., 2008; Sun et al., 2005). These conflicting reports might be due to differences in experimental conditions or cell types used (e.g., PBMC or CD4$^+$ T cells). Interestingly, we found that, unlike PBMC and CD4$^+$ T cells, human β defensins did not affect HIV infectivity of LCs (Figure 3). Although no significant differences were detected, hBD2 and HD5 tended to decrease HIV infectivity of LCs. In addition, consistent with a previous finding that LL-37 inhibits HIV replication in CD4$^+$ T cells (Bergman et al., 2007), we found that LL-37 significantly inhibited HIV infectivity in DCs, probably through downregulation of surface DC-SIGN expression (Figure 6). By contrast, LL-37 upregulated surface expression of CD4 and CCR5 in LCs, and upregulation strongly correlated with the increased R5 HIV entry and infection within these cells (Figure 4 and Figure 5). Thus, these findings clearly indicate that the effects of AMPs on HIV infectivity are differentially regulated depending on the target cell type.

We found that HSV-2 infection in epithelial cells induced the production of soluble LL-37 that had potent enhancing effects on HIV infectivity in LCs. siRNA-mediated interference of LL-37 transcription blocked, at least in part, increased HIV infectivity. We hypothesize that when HSV-2 infection occurs in genital mucosa, LL-37 produced by HSV-infected epithelial cells augments HIV susceptibility of LCs, thereby leading to enhanced sexual transmission of HIV. Notably, a recent study has shown that cervicovaginal levels of LL-37 were associated with increased HIV acquisition in Kenyan sex workers (Levinson et al., 2009), a clinical finding that supports our hypothesis. Thus, HSV-2 can mediate both direct enhancing effects on HIV susceptibility in LCs as well as indirect enhancing effects via LL-37 production as shown here. These results further our understanding of the complex biologic events that occur during the early stages of sexual transmission of HIV.

EXPERIMENTAL PROCEDURES

Reagents

Cells were stimulated with synthetic AMPs (Peptide Institute) for 24 hr at the following concentrations: α defensin-5 (50 μg/ml), β defensin-1 (50 μg/ml), β defensin-2 (50 μg/ml), β defensin-3 (5 μg/ml), β defensin-4 (50 μg/ml), and LL-37 (50 μg/ml). Recombinant human (rh) TNF-α (5 μg/ml, R&D Systems) was used as a positive control in some experiments. Anti-TNF-α neutralizing mAbs (clone; MABTNF-A5) were purchased from BD PharMingen and used at a final concentration of 1 μg/ml.

Cell Preparation

NHEKs were purchased from Kurabo and cultured with EpiLife supplemented with insulin (10 μg/ml), rhEGF (epidermal growth factor, 0.1 ng/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), and bovine pituitary extract (0.4% V/V) (EpiLife-KG2 medium, all from Kurabo) in a humidified atmosphere with 5% CO2 at 37°C.

mLCs and mDCs were cultured from adult plastic-adherent PBMCs as described previously (Kawamura et al., 2001). Briefly, monocytes were isolated by depletion of magnetically labeled nonmonocytes (Monocyte Isolation Kit II, Miltenyi Biotec) from plastic-adherent PBMCs obtained from healthy blood donors. Monocytes were cultured in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated FBS (Cell Culture Technologies), 100 U/ml penicillin (GIBCO BRL), 100 μg/ml streptomycin (GIBCO BRL), 2 mM L-glutamine (GIBCO BRL) (complete medium) supplemented with 1,000 U/ml rhGM-CSF (R&D Systems), 1,000 U/ml rhIL-4 (R&D Systems), and with mLCs or without mDCs 10 ng/ml human platelet-derived TGF-β1 (R&D Systems) for 7 days. Since we have previously found the expression levels of E-cadherin$^+$ cells and langerin$^+$ cells in mLCs to be approximately 90% and 35%, respectively (Kawamura et al., 2001), cell sorting was performed at day 7 to isolate highly purified langerin-positive mLCs followed by staining with anti-langerin mAb (Immunotech), as previously described (Ogawa et al., 2009). Alternatively, mLCs were identified by gating langerin-positive cells in flow cytometric analyses.

HSV-2 Exposure of Cells In Vitro and Skin Explants Ex Vivo

Purified, pelleted, and titered HSV-2 G strain (stock at 10^8 PFU/ml) was purchased from Advanced Biotechnologies. HSV-2 strain 186 (stock at 1.5×10^7 PFU/ml) was a gift from Yukihiro Nishiyama (Nagoya University Graduate School of Medicine, Nagoya, Japan). A total of 2×10^5 mLCs or mDCs, or 5×10^6 NHEK, were cultured with different concentrations of HSV-2 (10^{4-6} PFU) at 37°C, and then washed three times. In some experiments using the supernatants from NHEKs treated by HSV-2, culture supernatants

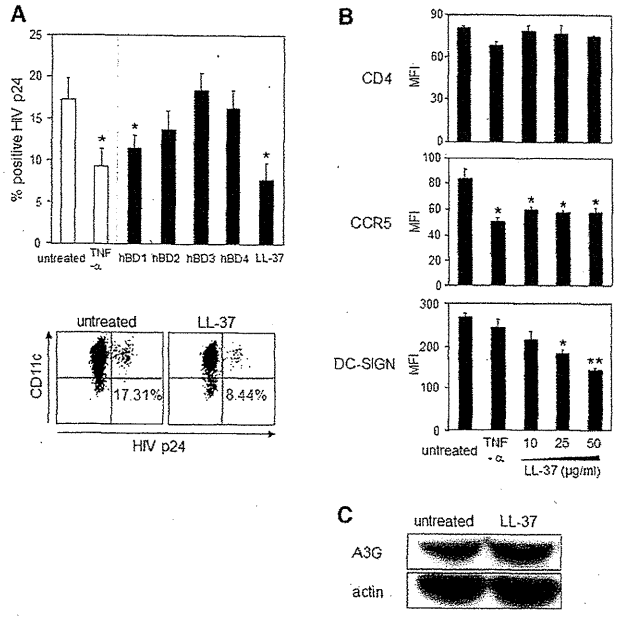

Figure 6. LL-37 Decreases HIV Susceptibility in mDCs

(A) mDCs were stimulated with the indicated AMPs or rhTNF-α 24 hr prior to HIV exposure. To determine HIV infection levels, mDCs were collected 7 days after HIV exposure, and HIV $p24^+$ cells were assessed in $CD11c^+$ mDCs. Representative flow cytometric analyses are shown.

(B) mDCs were stimulated with TNF-α or LL-37 at the indicated concentrations for 24 hr. The expression of CD4, CCR5, and DC-SIGN was assessed by flow cytometry.

(C) The expression of A3G was determined by western blot analysis. Results are shown as means ± SD (n = 3) (*$p < 0.05$; **$p < 0.01$). All data shown represent at least two separate experiments.

containing HSV-2 were filtered by PALL Acrodisc 32 mm Syringe Filter with 0.1 μm Supor Membrane to remove viruses. For control infection, the same batch of virus was inactivated at 56°C for 10 min and the same volume as the active virus was added to cells. For exposure of epithelial tissue explants, 50 μl droplets containing different concentrations of HSV-2 were placed on the inside surfaces of sterile plastic culture dish covers. Explants were draped over droplets with the basal epithelial cell surface facing downward. Virus and explants were incubated together in this manner at 37°C in a humidified 5% CO_2 environment for 1 hr, and then washed three times with cold PBS.

HIV Infection of Cells In Vitro and Skin Explants Ex Vivo

Purified, pelleted, and titered HIV-1Ba-L, an R5 HIV laboratory isolate (stock at $TCID_{50}$ of $10^{7.17}$/ml and 1.8×10^{10} virus particles/ml), was purchased from Advanced Biotechnologies. Molecular clones R5 HIV primary isolates (JR-FL and AD8) were prepared as described previously (Koyanagi et al., 1997; Theodore et al., 1996). Briefly, 293T cells were transfected with 30 μg of HIV-1 proviral DNA. One day after transfection, the medium was replaced with fresh RPMI 1640 medium supplemented with 10% FCS, and then 2 days later, the viruses were recovered, filtered through a membrane (pore size, 0.22 mm), and assayed for HIV-1 p24 gag content by ELISA. The titer of each virus stock was determined by endpoint titer determination of 3-fold limiting dilution in triplicate on PHA-activated PBMC from a single donor. Aliquots of the viral stocks ($TCID_{50}$ of 9,004,929/ml; JR-FL and 7,746,147/ml; AD8) were stored at minus 80°C until use. For some experiments, 2×10^5 mLCs and mDCs were preincubated with various agonists, inhibitors, HSV-2, or the supernatants from NHEKs treated by HSV-2, and then HIV-1Ba-L at a 1/100 final dilution or R5 HIV primary isolates (JR-FL and AD8) at $TCID_{50}$ of 10^5/ml was added for 2 hr at 37°C, as described previously (Kawamura et al., 2001). After incubation, cells were harvested, washed three times in washing medium (HBSS containing 10% heat-inactivated FBS), resuspended in complete medium supplemented with GM-CSF and IL-4, and cultured for 7 additional days at the same cellular concentration. HIV-infected cells were assessed by HIV p24 intracellular staining. Because the variability in the infection levels was most likely due to the *CCR5* heterogeneity in the donors, HIV infection levels with mLCs and mDCs obtained from different donors were not directly compared. Instead, HIV infection levels were expressed as a normalized percent of the positive cells for HIV p24 by using a calculated fold difference as compared with the mean percent of the positive cells for HIV p24 in untreated cells (Figure 3A). In some experiments, 2×10^4 HIV-infected mLCs or mDCs were cocultured with 2×10^6 allogeneic $CD4^+$ T cells for 12 days, and supernatants were harvested every third day and examined for HIV p24 protein content by ELISA (ZeptoMetrix) according to the manufacturer's instructions.

Epithelial sheets were obtained from suction blister roofs from HIV-negative healthy donors. For infection of epithelial tissue explants, 50 μl droplets containing HIV-1Ba-L at a 1/100 final dilution were placed on the inside surfaces of sterile plastic culture dish covers, as described previously (Kawamura et al., 2000). Explants were draped over droplets with the basal epithelial cell surface facing downward. Virus and explants were incubated together in this manner at 37°C in a humidified 5% CO_2 environment for 2 hr. Explants were washed in three separate wells in 6-well plates containing sterile PBS and then floated with the basal epithelial cell sides down in 12-well plates containing 2 ml of complete medium, without exogenous stimulants or cytokines. The emigrating cells from the epidermal sheets were collected 3 days after the HIV exposure. In some experiments, epidermal cell suspensions were prepared by limited trypsinization of epidermal sheets, as described previously (Miller et al., 2011a).

Pseudotyped Virus Infection and Luciferase Assay

To prepare pseudotyped viruses with Env from either HIV-1 (IIIB, JR-FL, or VSV), 293 T cells were cotransfected with the Env expression plasmid DNA, pLET, pJRFLenv, or pMD.G, respectively, and with pNLLuc (an Env-defective HIV-1NL4-3 carrying the luciferase gene) as described previously (Sato et al., 2008). The culture supernatants were harvested and then filtrated to produce virus solutions at 48 hr posttransfection. To measure the infectivity of Env-pseudotyped virus, mLCs were incubated with JR-FL Env- or IIIB Env-pseudotyped virus, containing 20 ng of p24CA, or VSV envelope glycoprotein-pseudotyped virus, containing 0.5 ng of p24CA, for 72 hr. The Picagene luciferase assay kit (Toyo Ink) was used to perform luciferase assays, following the manufacturer's protocols. Activity was measured with a 1420 ARVOSX multilabel counter (Perkin Elmer) and normalized to the protein content of each lysate, measured with a Coomassie (Bradford) protein assay kit (Pierce).

Flow Cytometry

Single-cell suspensions were stained using the following anti-human mAb: anti-CD83 (BD Biosciences-PharMingen), anti-CD86 (BD Biosciences-PharMingen), anti-CD4 (Beckman Coulter), anti-CCR5 (R&D), anti-DC-sign (R&D), anti-CCR7 (R&D) directly conjugated to FITC, anti-langerin (Immunotech) directly conjugated to PE, and anti-CD11c (Becton Dickinson) directly conjugated to allophycocyanin. Cells were incubated with Abs for 30 min at 4°C and then washed three times in staining buffer and examined by FACScaliber using propidium iodide (Sigma) to exclude the dead cells in the surface staining.

To specifically identify HIV- or HSV-infected cells on a single-cell level, HIV p24 or HSV gD intracellular staining was performed, respectively. Epidermal LCs, mLCs, and mDCs were collected at the indicated days after HIV exposure and then washed three times in staining buffer, and then incubated with 10 μg/ml allophycocyanin-conjugated mouse anti-human CD11c mAb, and with mLCs or without mDCs PE-conjugated mouse anti-human langerin mAb and for 30 min at 4°C. Cells were then washed three times in staining buffer and fixed and permeabilized with Cytofix/Cytoperm reagents (BD Biosciences-PharMingen) for 20 min at 4°C. Cells were then washed three times in Perm-Wash (BD Biosciences-PharMingen), incubated with FITC- or PE-conjugated mouse anti-HIV p24 mAb (Beckman Coulter) and/or FITC-conjugated mouse anti-HSV gD mAb (Argene) diluted for 30 min at 4°C, and washed three times in Perm-Wash, with the quantified numbers of HIV- or HSV-infected cells determined by FACScaliber.

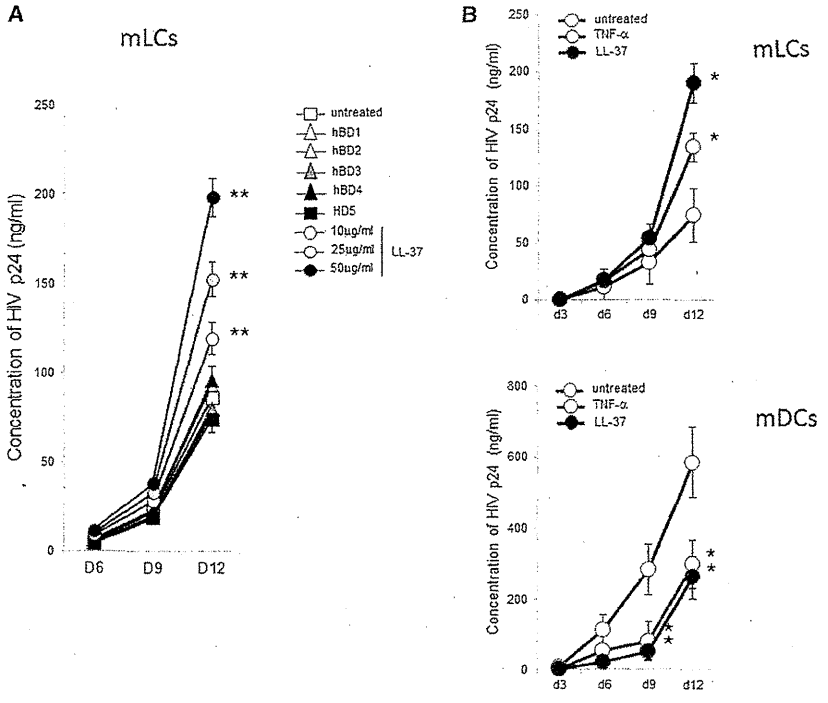

Figure 7. LL-37 Enhances HIV Transmission from mLCs to T Cells

mLCs (A) or mLCs and mDCs isolated from the same donor (B) were stimulated with the indicated AMPs or rhTNF-α 24 hr prior to HIV exposure. HIV-infected mLCs or mDCs were cocultured with allogeneic $CD4^+$ T cells, and p24 protein levels in culture supernatants were assessed by ELISA on the indicated days. Results are shown as means plus or minus SD ($n = 3$). $^*p < 0.05$; $^{**}p < 0.01$. All data shown represent at least two separate experiments.

RNA Interference Using siRNA

The delivery of siRNA into NHEKs was performed by DharmaFECT 3 siRNA Transfection Reagent (Dharmacon). Cells were transfected with siRNAs at a final concentration of 50 nM. The siRNAs used in this study were ON-TARGETplus nontargeting pool (Dharmacon #D 001810–10) for control siRNA and ON-TARGETplus SMARTpool siRNA Human CAMP (Dharmacon #L-019790–00) for LL-37 siRNA.

Real-Time Quantitative RT-PCR Analysis

Relative mRNA expression was determined by real-time PCR using an ABI PRISM 5500 Sequence Detection System (Applied Biosystems) with SYBR Green I dye (QIAGEN) according to the manufacturer's instructions. Total RNA was isolated using TRIzol (Invitrogen Life Technologies), and cDNA was synthesized using the SuperScript system (Invitrogen Life Technologies). Primers corresponding to human α defensin-5, defensin-6, human β defensin-1, β defensin-2, β defensin-3, β defensin-4, LL-37, and GAPDH were designed by Takara Bio, Inc. Cycle threshold numbers (Ct) were derived from the exponential phase of the PCR amplification. Fold differences in the expression of gene x in the cell populations y and z were derived by 2k, where $k = (Ctx - CtG3PDH)y - (Ctx - CtG3PDH)z$.

ELISA

NHEKs were exposed to live HSV-2 (10^6 PFU) or heat-inactivated HSV-2 for 1 hr, and then washed three times. Following culture in medium for the indicated days, the culture supernatants were collected after centrifugation and stored at −80°C for LL-37 and TNF-α measurement. The concentration of human LL-37 (Hycult biotechnology) and TNF-α (R&D Systems) in the culture supernatants was measured by ELISA. For measurement of HIV p24 protein levels, supernatants were collected, inactivated with Triton X-100 (Sigma-Aldrich; 2% final concentration), and kept frozen until measurements of HIV p24 protein levels were performed by ELISA (ZeptoMetrix).

Western Blot Analysis

Proteins of the cells were extracted using 15 min incubation in complete lysis buffer containing a protease inhibitor. Equal amounts of protein were separated by SDS-PAGE and transferred onto a transfer membrane (Daiichikagaku). Western blot was performed in order to detect hCAP18 (2.0 μg/ml, Abcam), KLK5 (2.0 μg/ml, R&D Systems), LL-37 (2.0 μg/ml, Santa Cruz), A3G (2.5 μg/ml, Abcam), and SAMHD1 (2.0 μg/ml, Abcam). Blots were incubated with the HRP-linked secondary antibody. Analyses were performed using the HRP western blot detection system (Pierce), and band intensities were calculated using ImageJ software.

Statistical Analyses

Significant differences between experimental groups were analyzed by Student's t test (one-tailed). p values less than 0.05 were considered significant.

Study Approval

The Institutional Review Board of the University Hospital (University of Yamanashi, Yamanashi, Japan) approved the acquisition of human tissues, and informed consent was obtained from all skin donors.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article at http://dx.doi.org/10.1016/j.chom.2012.12.002.

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LL-37 Enhances HIV Infection of Langerhans Cells

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Deep-Sequencing Analysis of the Association between the Quasispecies Nature of the Hepatitis C Virus Core Region and Disease Progression

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Variation of core amino acid (aa) 70 of hepatitis C virus (HCV) has been shown recently to be closely correlated with liver disease progression, suggesting that the core region might be present as a quasispecies during persistent infection and that this quasispecies nature might have an influence on the progression of disease. In our investigation, the subjects were 79 patients infected with HCV genotype 1b (25 with chronic hepatitis [CH], 29 with liver cirrhosis [LC], and 25 with hepatocellular carcinoma [HCC]). Deep sequencing of the HCV core region was carried out on their sera by using a Roche 454 GS Junior pyrosequencer. Based on a plasmid containing a cloned HCV sequence (pCV-J4L6S), the background error rate associated with pyrosequencing, including the PCR procedure, was calculated as 0.092 ± 0.005/base. Deep sequencing of the core region in the clinical samples showed a mixture of "mutant-type" Q/H and "wild-type" R at the core aa 70 position in most cases (71/79 [89.9%]), and the ratio of mutant residues to R in the mixture increased as liver disease advanced to LC and HCC. Meanwhile, phylogenetic analysis of the almost-complete core region revealed that the HCV isolates differed genetically depending on the mutation status at core aa 70. We conclude that the core aa 70 mixture ratio, determined by deep sequencing, reflected the status of liver disease, demonstrating a significant association between core aa 70 and disease progression in CH patients infected with HCV genotype 1b.

Hepatitis C virus (HCV)-related liver disease gradually advances from chronic hepatitis (CH) to liver cirrhosis (LC) and to hepatocellular carcinoma (HCC) over 20 to 30 years (1). However, the rates of disease progression differ: some patients develop HCC over several years, while others show persistently normal alanine aminotransferase (PNALT) levels for decades, and the cause of the difference remains poorly understood.

The involvement of viral and host factors in the progression of liver disease and hepatocarcinogenesis is complex (2). With regard to viral factors, the relationship between the viral core region and disease progression in HCV genotype 1b infection has attracted clinical attention. Specifically, it has been reported that the core amino acid (aa) 70 residue, identified as a variable related to the outcome of interferon (IFN) therapy (3), is closely associated with the progression of hepatitis and hepatocarcinogenesis in Japan and North America (4–7). Those previous studies and our own have reported that core aa 70 variation is strongly linked to carcinogenesis and that substitutions in the core aa 70 region aggravate hepatitis and heighten the risk of hepatocarcinogenesis during the clinical course, corroborating the relationship between the status of the core region and disease progression (8, 9).

With regard to host factors, a genomewide association study (GWAS) has recently shown that single nucleotide polymorphisms (SNPs) around the interleukin 28B (IL28B) gene (rs12979860 and rs8099917), encoding the type III IFN IFN-λ3, are strongly correlated with the outcomes of therapy with pegylated IFN-α plus ribavirin for chronic hepatitis C (CH-C) (10–13). Interestingly, in contrast to the core region, consensus has not been reached as to the relationship between disease progression and the IL28B SNP, which is associated with IFN resistance (14–17). Previously, we reported that there was no correlation between the onset of HCC and the IL28B SNP (9). However, it was reported that the IL28B rs8099917 TG/GG allele was markedly correlated with the presence of Q (glutamine) or H (histidine) instead of the R (arginine) residue at core aa 70, while the IL28B rs8099917 TT allele was correlated with core aa 70R (8). Moreover, the core amino acid R70Q/H change occurs more often in patients with IL28B rs8099917 TG/GG than in those with IL28B rs8099917 TT (9). In this manner, the contributions of the core aa 70 residue and the IL28B SNP, which were found to be IFN sensitivity factors, to the progression of liver disease have gradually been elucidated.

HCV exists in a host as a swarm of variants, known as a "quasispecies," and this quasispecies nature has been considered to play a critical role in pathogenesis (18). However, detailed analysis has been technically difficult, and the clinical significance has not been clarified in detail thus far. Considering the observation of core aa 70 gene changes over time, it is assumed that a variety of core aa 70 isolates may exist as a quasispecies, and this could be related to pathogenesis as described above.

Recently, deep-sequencing technology has advanced rapidly and has enabled us to analyze viral quasispecies in association with the status of the disease (19–22). In this study, we investigated how the quasispecies of the HCV core gene, either at the hot spot of the core aa 70 residue or in the almost-entire core gene, is created and is involved in disease progression in patients with CH-C.

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TABLE 1 Patient characteristics classified by disease progression

Characteristic[a]	Value for patients with:			P
	CH ($n = 25$)	LC ($n = 29$)	HCC ($n = 25$)	
No. male/female	14/11	10/19	12/13	0.278
Age (yr) (mean ± SD)	63.4 ± 14.6	66.5 ± 9.2	68.4 ± 8.2	0.539
Platelets (10^{-4}/mm^3) (mean ± SD)	16.1 ± 4.7	9.8 ± 3.9	11.1 ± 5.1	<0.001
Albumin (g/dl) (mean ± SD)	4.4 ± 0.3	3.9 ± 0.6	3.5 ± 0.5	<0.001
γ-GTP (IU/liter) (median [range])	53.5 (12–230)	38.0 (12–108)	40.8 (15–110)	0.845
T. chol. (mg/dl) (mean ± SD)	161 ± 28	148 ± 30	140 ± 28	0.053
HCV RNA (kIU/ml) (median [range])	7,047 (501–19,953)	5,369 (126–25,119)	8,421 (110–25,119)	0.288
Alpha-fetoprotein (ng/ml) (median [range])	5.0 (1.1–16.5)	32.2 (1.0–252.6)	614.8 (1.9–13,418)	<0.001
AST (IU/liter) (mean ± SD)	42.4 ± 17.8	51.1 ± 23.5	59.1 ± 28.2	0.046
ALT (IU/liter) (mean ± SD)	46.6 ± 23.1	43.9 ± 29.3	60.7 ± 52.7	0.263
No. with R/(Q/H) at core aa 70[b]	19/6	12/17	8/17	0.005
No. with L/(M/C) at core aa 91[b]	17/8	19/10	15/10	0.834
No. of ISDR mutations (median [range])[b]	0.8 (0–6)	1.2 (0–7)	0.9 (0–8)	0.799
No. of IRRDR mutations (median [range])[b]	4.9 (1–10)	4.7 (2–9)	5.2 (1–12)	0.962
No. with TT/non-TT at IL28B SNP (rs8099917)	18/7	17/12	14/11	0.458
No. without/with a history of interferon therapy	14/11	16/13	15/10	0.933

[a] T. chol., total cholesterol; AST, aspartate transaminase; ALT, alanine aminotransferase.

[b] Core aa 70, core aa 91, the interferon sensitivity-determining region (ISDR), and the interferon-ribavirin resistance-determining region (IRRDR) were dominant viral sequences determined by direct sequencing.

PATIENTS AND METHODS

Patients. The subjects were 79 patients persistently infected with HCV genotype 1b who were followed up at Yamanashi University Hospital. The patients all fulfilled the following criteria: (i) they were negative for hepatitis B surface antigen; (ii) they had no other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease; (iii) they were free of coinfection with human immunodeficiency virus; and (iv) signed consent was obtained for the study protocol. The study protocol had been approved by the Human Ethics Review Committee of Yamanashi University Hospital and conformed to the ethical guidelines of the Declaration of Helsinki.

The breakdown was as follows: 25 patients with CH, 29 with LC, and 25 with HCC. The patients' clinical backgrounds, including histories of interferon-based antiviral therapy, are shown in Table 1. Deep-sequencing analysis was performed using serum samples taken at the most recent visit from patients with chronic hepatitis or liver cirrhosis and at the first diagnosis of HCC from patients with HCC. A direct-sequencing method, which determines the dominant viral sequence, was performed as described previously (9) to determine the dominant viral sequences of the core region, the interferon sensitivity-determining region (ISDR), and the interferon-ribavirin resistance-determining region (IRRDR) from the serum of each patient.

Deep sequencing. Deep sequencing of the viral core region was performed for each of 79 patients. Briefly, RNA was extracted from the stored sera of these patients and was reverse transcribed to cDNA. Then two-step nested PCR was carried out with primers specific for the core region of the HCV genome (23). The primers for the second-round PCR had barcodes attached, were 10 nucleotides (nt) long, and differed for each sample, so that PCR products from each sample were identifiable (see Table S1 in the supplemental material). After the band densities of the PCR products were quantified using a Bioanalyzer (Agilent Technologies, Palo Alto, CA), the concentrations of the samples were adjusted to a common value, and pooled samples were prepared. Libraries were then subjected to emulsion PCR, the enriched DNA beads loaded onto a picotiter plate, and pyrosequencing carried out with a Roche GS Junior/454 sequencing system using titanium chemistry (Roche, Branford, CT). In order to determine the error rate of the procedure, deep sequencing was carried out under similar conditions with a plasmid containing a cloned HCV sequence (pCV-J4L6S) (24). Amplicon Variant Analyzer software, version 2.5p1 (Roche), was used for analysis.

A dominant sequence of the core region for each patient was deposited in GenBank. Although the study amplified 499 nucleotides, from the 25th to the 523rd nucleotide of the core region, by PCR (Fig. 1), information for only 459 of the 499 nucleotides was uploaded for each patient, since some minor PCR amplicons obtained by deep sequencing did not include the full 499 nucleotides.

Phylogenetic tree analysis. Phylogenetic trees were constructed from the sequences by using the neighbor-joining method with BioEdit and MEGA5.05, and bootstrapping was performed with 1,000 replicates (25). In constructing phylogenetic trees, the three bases of the core codon 70 were removed in the analysis of all trees, since the mutation rate of other parts of the core region is known to be rather low, and it was possible that the influence of the core aa 70 mutations might be overestimated in the phylogenetic trees. In addition, using genetic distance data obtained from the phylogenetic analysis, the genetic distances between every two HCVs with core aa 70R, between every two HCVs with a residue other than R at core aa 70 (core aa 70non-R), and between every two HCVs with different residues at core aa 70 (one HCV with R and one with a non-R residue) were also compared statistically in order to reveal the genetic associations among those HCV core subgroups.

Statistical analysis. Statistical differences in the parameters, including all available patients' demographic, biochemical, hematological, virological, and SNP data in the three groups (CH, LC, and HCC), were determined using the Kruskal-Wallis test. The Mann-Whitney U test was used for statistical differences in numerical variables between two groups. Trends for categorical data

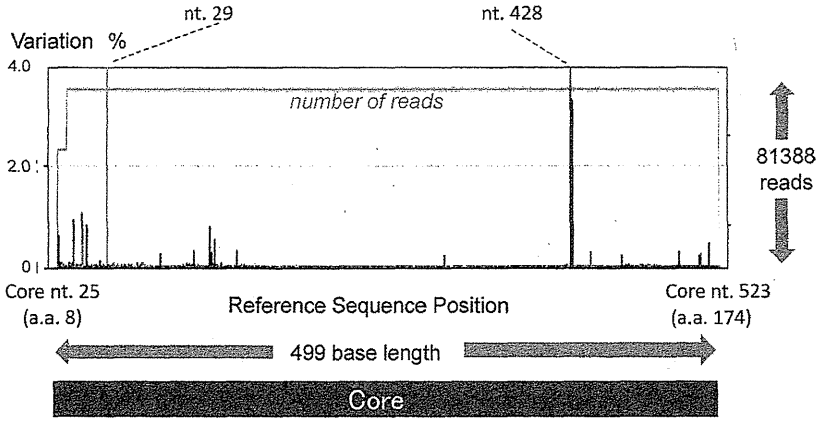

FIG 1 The core region of pCV-J4L6S (genotype 1b) from the 25th to the 523rd nucleotide, and from the 9th to the 174th codon, was subjected to deep sequencing, and the background error rate of pyrosequencing was calculated. In order to show rare background errors, those errors with low percentages are magnified.

were evaluated using the Cochran-Armitage trend test. All *P* values of <0.05 by the two-tailed test were considered significant in comparisons of genetic distances.

Nucleotide sequence accession numbers. The dominant sequences of the core regions from the patient samples have been deposited in GenBank under accession numbers AB822372.1 to AB822459.1.

RESULTS

Calculation of background errors in deep sequencing. First, the background error rate of pyrosequencing was calculated with a plasmid containing a cloned HCV sequence (pCV-J4L6S). Figure 1 shows the results of deep sequencing from the 25th to the 523rd nucleotide of the core region (499 nt) in pCV-J4L6S. Among 81,388 reads, each 499 nt long, the maximum error rate was 99.14% at the 428th base, and the next highest error rate was 64.17% at the 29th base. A six-C homopolymer region ending at the 428th base was read as a five-C homopolymer and a five-A homopolymer region ending at the 29th base was read as a four-A homopolymer in most of the obtained sequences; these homopolymer sequences are a weak point of pyrosequencing (26, 27).

A base appearing six times consecutively was the longest sequence of identical repeated nucleotides and was found only at the 428th nt position in the core region of pCV-J4L6S. Five consecutive bases were found at two sites (the 336th and 436th nt) in addition to the 29th nt position, but this error (i.e., the miscounting of homopolymer length) occurred only at the 29th base closest to the end of the sequence. Excluding the 428th and 29th nt positions, the error rate was ~1% or lower, as shown in Fig. 1. There was no single nucleotide error in the codons for aa 70 and aa 91in the repeated control experiments. From repeated deep sequencing of the plasmid, the overall nucleotide error rate was calculated as 0.092 ± 0.005 (mean ± standard deviation [SD])/base. Based on this analysis, a mixture of bases detectable above the background error of 0.102% (mean background error rate + 2 SDs) was defined as a real mixture.

Baseline characteristics. The baseline characteristics of the 79 patients are shown in Table 1. The values for viral factors core aa 70 and aa 91, NS5A-ISDR, and NS5A-IRRDR are the results of the direct-sequencing study. As shown in Table 1, the results for the variables platelets, albumin, alpha-fetoprotein, and core aa 70 differed significantly according to disease progression. On the other hand, no difference was observed in core aa 91 and IL28B SNP (rs8099917) according to disease progression.

Quasispecies nature of core amino acid 70 and disease progression. Deep sequencing of the core region was carried out with a variety of clinical samples. Simultaneous analysis was carried out using the barcoded primers, and approximately 950 reads were obtained per sample (Table 2). When the analysis was focused on core aa 70, the proportion of non-R (Q/H) sequences increased as disease severity advanced from CH to LC to HCC, as shown in Fig. 2A and Table 3 ($P = 0.018$). When a mixture of 0.102% or more was defined as a real mixture, deep sequencing showed the presence of a mixture at core aa 70 in 71 of the 79 patients (89.9%).

The relationship between disease progression and the occurrence of a quasispecies was also analyzed at the codon for core aa 91, which has also been reported to be associated with the outcomes of IFN therapy and the occurrence of HCC. As with core aa 70, a quasispecies was recognized at this site, and mixtures were observed in most patients. However, in contrast to the core aa 70 codon, there was no clear relationship with disease progression (Fig. 2B and Table 3).

Figure 2C and D show the correlation between mixtures in the core aa 70 and 91 regions and IL28B SNPs. As shown in Fig. 2C and Table 4, the proportion of mutations in the core aa 70 codon was highly dependent on the IL28B SNP (*P*, <0.005 [Table 4]). Such a relationship was also found between the proportion of mutations in core aa 91 and IL28B SNPs (Fig. 2D and Table 4), although its significance was rather weaker (*P*, 0.010).

TABLE 2 Amplicon read numbers obtained by deep sequencing of samples from 79 patients

Group	No. of patients	Total no. of reads	Avg no. of reads ± SD (range)/sample
CH	25	22,365	894.6 ± 222.8 (367–1,486)
LC	29	28,537	982.5 ± 258.1 (660–1,528)
HCC	25	24,284	971.4 ± 242.5 (405–1,749)
Total	79	75,186	951.7 ± 240.9 (367–1,749)

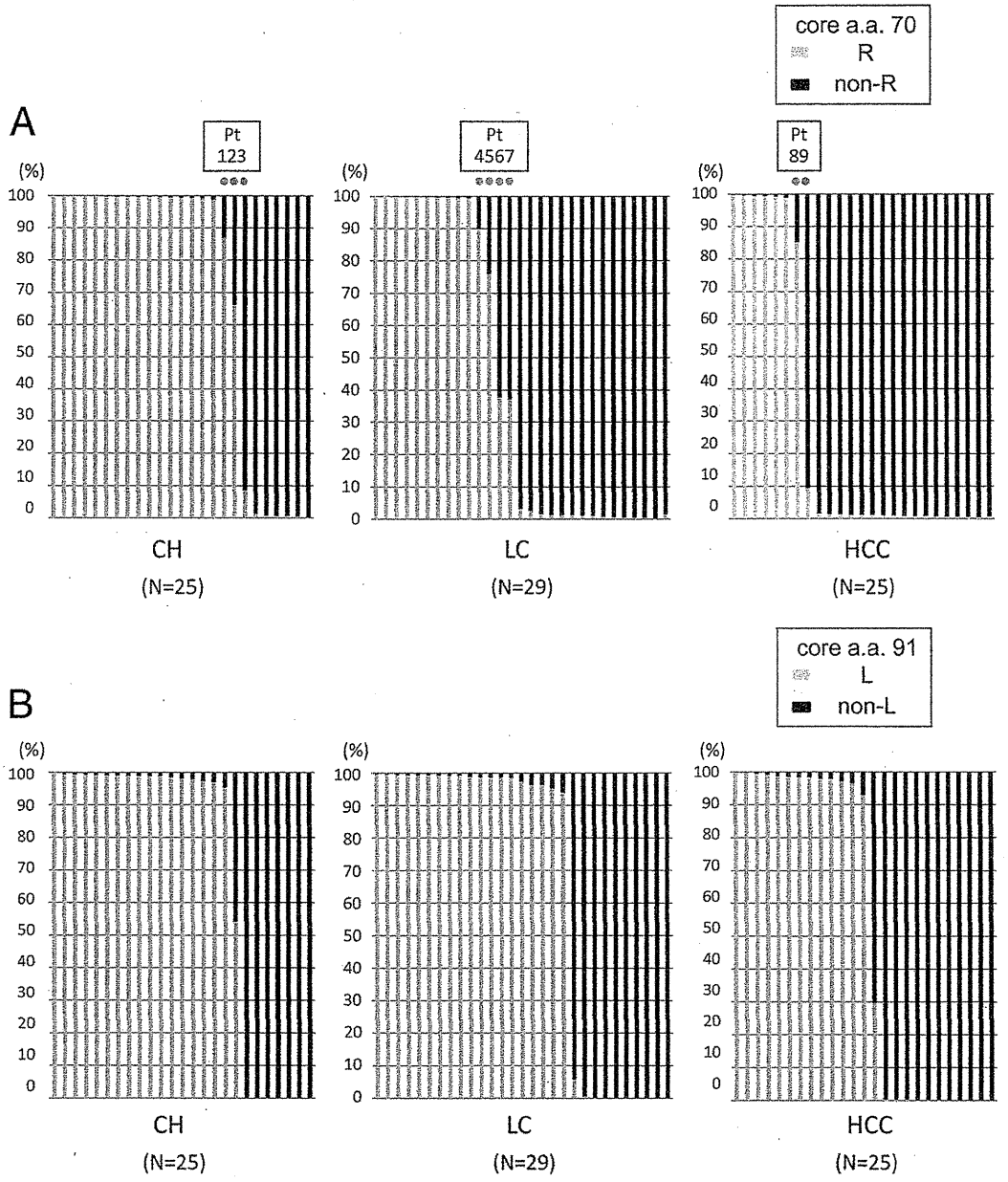

FIG 2 The core regions of the HCV genomes from 79 patients persistently infected with HCV genotype 1b (25 patients with chronic hepatitis [CH], 29 with liver cirrhosis [LC], and 25 with hepatocellular carcinoma [HCC]) were subjected to deep sequencing. Each bar represents the result for a single patient. At the specific locations of core aa 70 and aa 91, no single nucleotide mutation was observed in the previous control plasmid experiment. (A) Disease stages and percentages of mutations at core aa 70. Nine dots indicate the nine patients with a high mixture rate (between 5% and 95%) at core aa 70 (R and non-R). (B) Disease stages and percentages of mutations at core aa 91. (C) IL28B SNP and percentages of mutations at core aa 70. (D) IL28B SNP and percentages of mutations at core aa 91.

Since direct sequencing has also shown an association of several sites other than core aa 70 and 91 with the occurrence of HCC (6), those sites were also investigated for such an association. However, there was no clear relationship between these sites and disease progression, except for G209A (core aa R70Q) (see Fig. S1 and Table S2 in the supplemental material).

Phylogenetic tree analysis of HCV core region focusing on the core aa 70 residue. Because it was clear that the core aa 70 quasispecies state was significantly associated with disease progression, our next interest was to determine how this single hot spot is correlated with the remainder of the (almost-entire) core region. Therefore, phylogenetic tree analysis was performed, and genetic distances among aa 70-associated core sequences were also compared statistically. In constructing all phylogenetic trees, the three bases of the core 70 codon were removed, since the mutation rate of other parts of the core region is known to be rather low, and it was possible that the influence of the core aa 70 mutations might be overestimated in the phylogenetic trees.

At first, to determine the associations among the remainder of the core sequences across different patients, a phylogenetic tree was constructed for all 79 patients using dominant core sequences obtained from each patient. In constructing the tree, two domi-

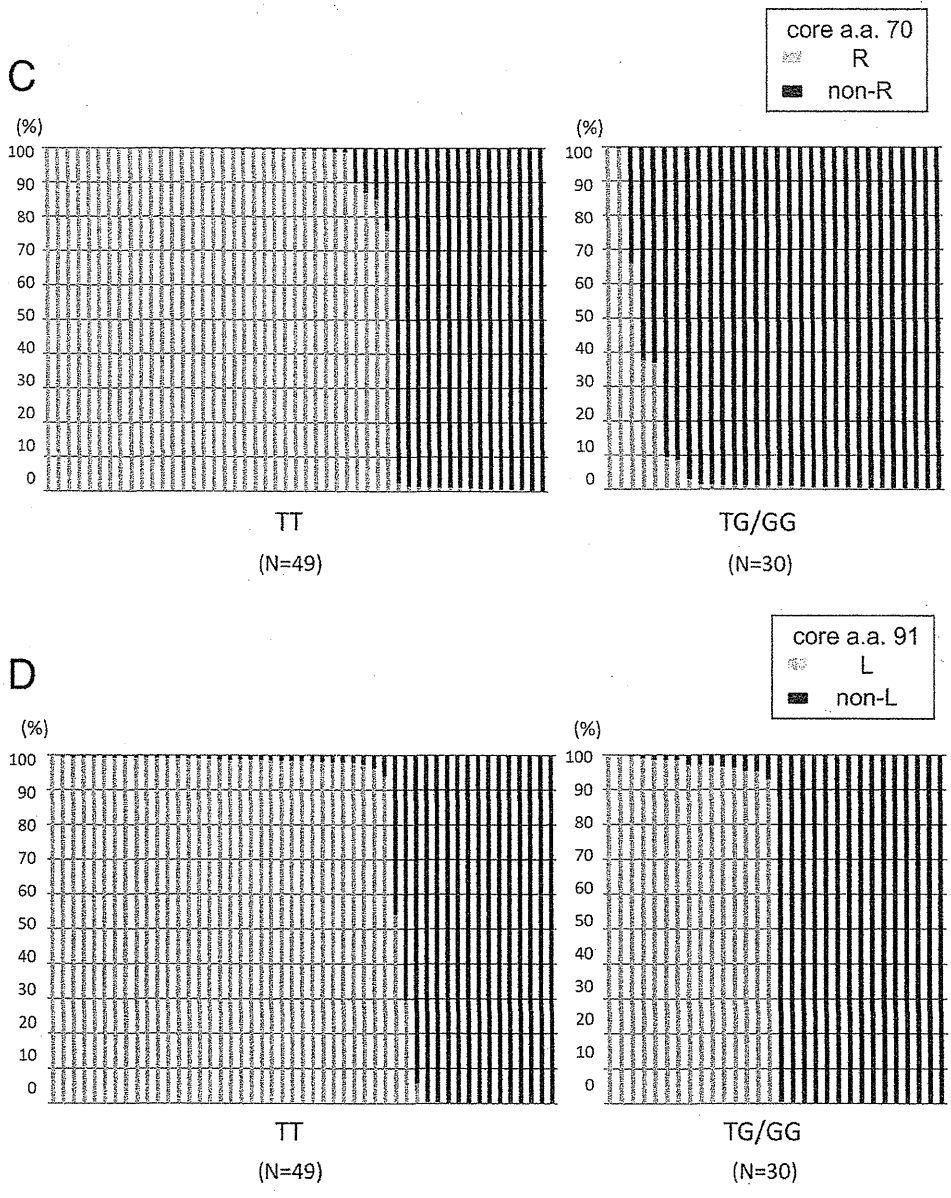

FIG 2 continued

nant sequences (the dominant core sequence in isolates with aa 70R and the dominant core sequence in isolates with aa 70non-R) were included in the analysis for each of the nine patients with high mixture rates (5% or more) of R and non-R at core aa 70 (Fig. 2A), while one dominant sequence each was included for other patients. As shown in Fig. 3A and Table 5, genetic distances calculated between every two core sequences with aa 70R (R-R) were significantly larger than those between two core sequences with aa 70non-R (non-R–non-R) or those between a

TABLE 3 Correlation between quasispecies composition and disease progression

Patient group (n)	Median % (range) with:	
	R at core aa 70[a]	L at core aa 91[b]
CH (25)	70.35 (0.00–100.00)	69.12 (0.00–99.40)
LC (29)	43.22 (0.24–100.00)	64.54 (0.00–99.50)
HCC (25)	28.20 (0.00–99.80)	52.23 (0.00–100.00)

[a] P, 0.018.
[b] P, 0.630.

TABLE 4 Correlation between quasispecies composition and IL28B SNP rs8099917

Group (sequence at IL28B SNP rs8099917)	Median % (range) with:	
	R at core aa 70[a]	L at core aa 91[b]
TT (n = 49)	68.15 (0.00–100.00)	68.24 (0.00–100.00)
TG/GG (n = 30)	12.64 (0.00–100.00)	48.76 (0.00–100.00)

[a] P, <0.005.
[b] P, 0.010.

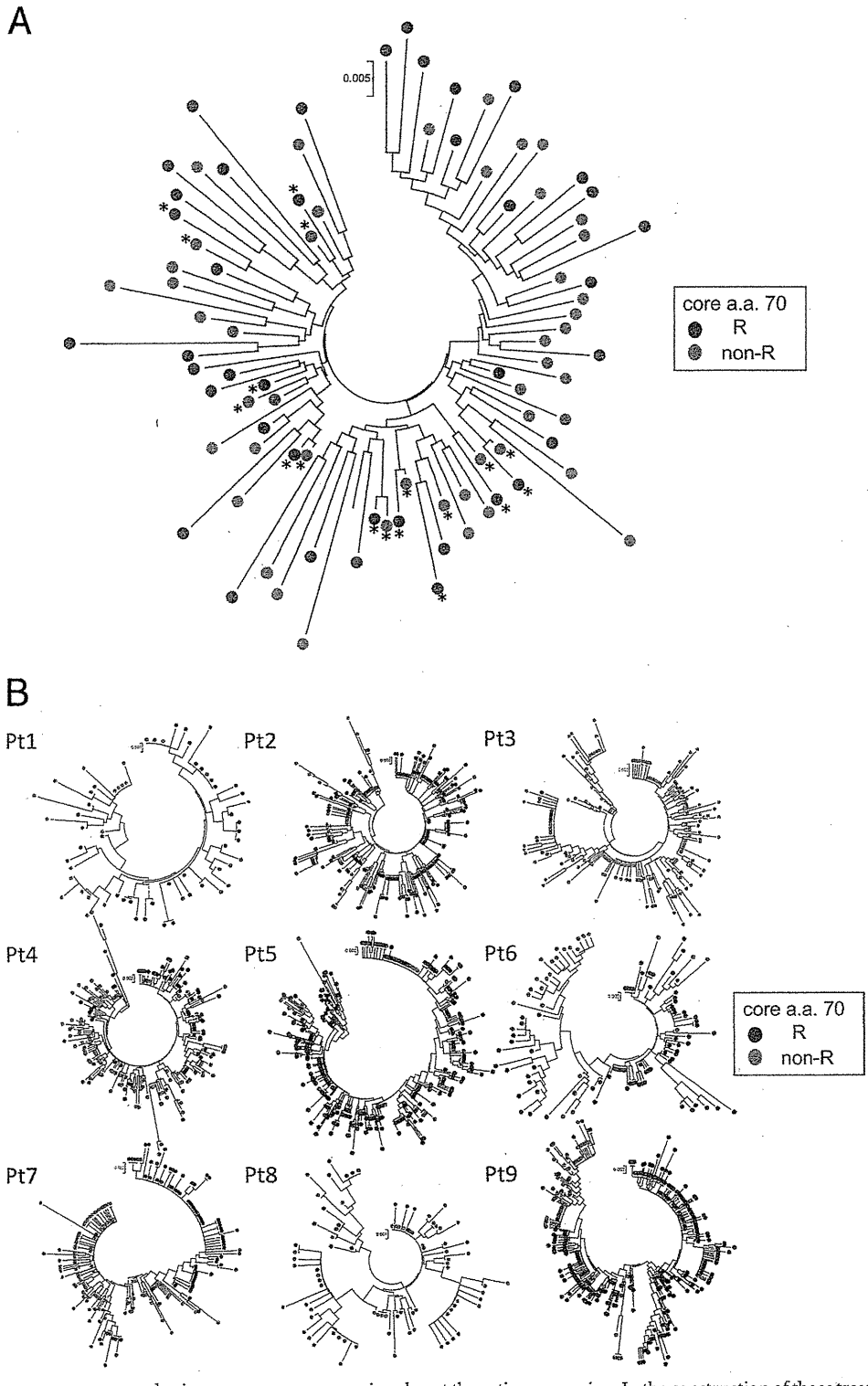

FIG 3 Phylogenetic trees were constructed using core sequences covering almost the entire core region. In the construction of those trees, the three bases of the core 70 codon were removed in the analysis of all 79 patients. Branches with core aa 70R are indicated by blue circles, while those with core aa 70non-R are indicated by pink circles. (A) Phylogenetic trees were constructed for all 79 patients by using dominant core sequences obtained from each patient. In the construction of the tree, two dominant sequences (a dominant core sequence in isolates with aa 70R and a dominant core sequence in isolates with aa 70non-R) were included in the analysis for each of the nine patients with high mixture rates (5% or more) of R and non-R at core aa 70 (Fig. 2A), while one dominant sequence each was included for other patients. (B) A phylogenetic tree of the core region was constructed for each patient with a high mixture rate (5% or more) of core aa 70R and core aa 70non-R. A total of nine patients were included in this analysis (patients 1 to 3 had CH; patients 4 to 7 had LC; and patients 9 and 10 had HCC). Pt, patient.

TABLE 5 Comparison of genetic distances among core subgroups related to aa 70 residues

Patient(s)[a]	Genetic distance (mean ± SD) between two core sequences[b]			Comparison of genetic distance measurements					
				Non-R–R vs non-R–non-R		Non-R–R vs R-R		Non-R–non-R vs R-R	
	Non-R–non-R	Non-R–R	R-R	Larger genetic distance	*P*	Larger genetic distance	*P*	Larger genetic distance	*P*
All ($n = 79$)	0.0349 ± 0.0101	0.0379 ± 0.0109	0.0401 ± 0.0113	Non-R–R	<0.001	R-R	<0.001	R-R	<0.001
CH									
Pt 1	0.0086 ± 0.0042	0.0098 ± 0.0037	0.0064 ± 0.0042	Non-R–R	<0.001	Non-R–R	<0.001	Non-R–non-R	<0.001
Pt 2	0.0097 ± 0.0048	0.0104 ± 0.0041	0.0087 ± 0.0038	Non-R–R	<0.001	Non-R–R	<0.001	Non-R–non-R	0.009
Pt 3	0.0107 ± 0.0058	0.0137 ± 0.0050	0.0034 ± 0.0022	Non-R–R	<0.001	Non-R–R	<0.001	Non-R–non-R	<0.001
LC									
Pt 4	0.0078 ± 0.0036	0.0103 ± 0.0038	0.0053 ± 0.0029	Non-R–R	<0.001	Non-R–R	<0.001	Non-R–non-R	<0.001
Pt 5	0.0118 ± 0.0090	0.0232 ± 0.0085	0.0159 ± 0.0170	Non-R–R	<0.001	Non-R–R	<0.001	No difference	0.991
Pt 6	0.0115 ± 0.0057	0.0121 ± 0.0055	0.0108 ± 0.0056	Non-R–R	<0.001	Non-R–R	<0.001	Non-R–non-R	<0.001
Pt 7	0.0141 ± 0.0085	0.0146 ± 0.0070	0.0136 ± 0.0067	Non-R–R	0.002	Non-R–R	<0.001	Non-R–non-R	<0.001
HCC									
Pt 8	0.0124 ± 0.0063	0.0225 ± 0.0060	0.0181 ± 0.0094	Non-R–R	<0.001	Non-R–R	<0.001	R-R	<0.001
Pt 9	0.0082 ± 0.0042	0.0162 ± 0.0047	0.0078 ± 0.0050	Non-R–R	<0.001	Non-R–R	<0.001	Non-R–non-R	<0.001

[a] Pt, patient.

[b] Non-R–non-R, comparison of two core sequences with residues other than R at aa 70; Non-R–R, comparison of a core sequence with a residue other than R at aa 70 and a core sequence with aa 70R; R-R, comparison of two core sequences with aa 70R. Genetic distances were calculated for all patients by using dominant sequences and for a single patient by using quasispecies sequences.

core sequence with aa 70R and a core sequence with aa 70non-R (non-R–R), demonstrating that core sequences with aa 70R were heterogeneous, while core sequences with aa 70non-R were homogeneous.

Next, to determine the association of the remainder of the core sequences in a single patient, phylogenetic trees were also constructed for each of the nine patients with high mixture rates (5% or more) of R and non-R residues at core aa 70 (Fig. 3B). As shown in Fig. 3B, HCV isolates with core aa 70R and those with core aa 70non-R formed distinctly clustered subgroups on the phylogenetic tree, according to the mutation status at core aa 70. Comparison of genetic distances also proved the finding that HCV isolates with core aa 70R and those with core aa 70non-R form distinctly clustered subgroups on the phylogenetic tree in a single patient, since genetic distances calculated between every two core sequences with aa 70R (R-R) or between every two core sequences with aa 70non-R (non-R–non-R) were significantly smaller than those between a core sequence with aa 70R and a core sequence with aa 70non-R (non-R–R). On the other hand, no significant difference was found when the genetic distance between two core sequences with aa 70non-R (non-R–non-R) and that between two core sequences with aa 70R (R-R) were compared in a single patient (Table 5).

Since the genetic relationships of the remainder of the core sequences were found to differ significantly according to the core aa 70 residue, we then investigated whether there are any common haplotypic sequences specific to each residue. In the comparison of dominant sequences in all 79 patients, most amino acid substitutions clustered in three amino acids (aa 70, aa 75, and aa 91) both in core sequences with aa 70R and in those with aa 70nonR, but no other substitutions specific to each core aa 70 residue were found (Fig. 4).

Quasispecies at core aa 70 and clinical characteristics. To clarify the association of the core aa 70 quasispecies with the clinical picture, levels of gamma-glutamyl transpeptidase (γ-GTP), albumin, platelets, and alpha-fetoprotein, as well as disease progression in the liver, were investigated for correlation with the core aa 70R/non-R mixture ratio. As shown in Fig. 5A and B, the values for these clinical parameters became significantly more abnormal as the proportion of non-R residues increased, showing that a high proportion of non-R residues at core aa 70 was significantly associated with disease severity and hepatocarcinogenesis.

DISCUSSION

This study examined, for the first time, the relationship between the progression of liver disease and the quasispecies nature of the HCV core region (already known to be associated with liver disease progression) by deep sequencing, with the focus on the core aa 70 residue. The analysis revealed that core aa 70 existed as a mixture of "mutant" Q/H (non-R) and "wild-type" R residues in most of the patients and that the proportion of mutant residues increased as liver disease advanced to LC and HCC. Meanwhile, phylogenetic analysis showed that the viral sequences of the almost-entire core region differed genetically depending on the status of core aa 70.

Before starting the analysis, we verified the rate of background error associated with the process of pyrosequencing by analyzing the control plasmid pCV-J4L6S (Fig. 1). Homopolymers of repeated bases, a weak point of pyrosequencing, were generated at two sites, with the same base appearing five and six times. The overall mutation rate at other sites was 0.092% ± 0.005%, and a mutation rate of 0.102% (mean + 2 SDs) or higher was defined as significant in the analysis, in order to avoid detecting background errors.

We focused our analysis on the quasispecies state of core aa 70,

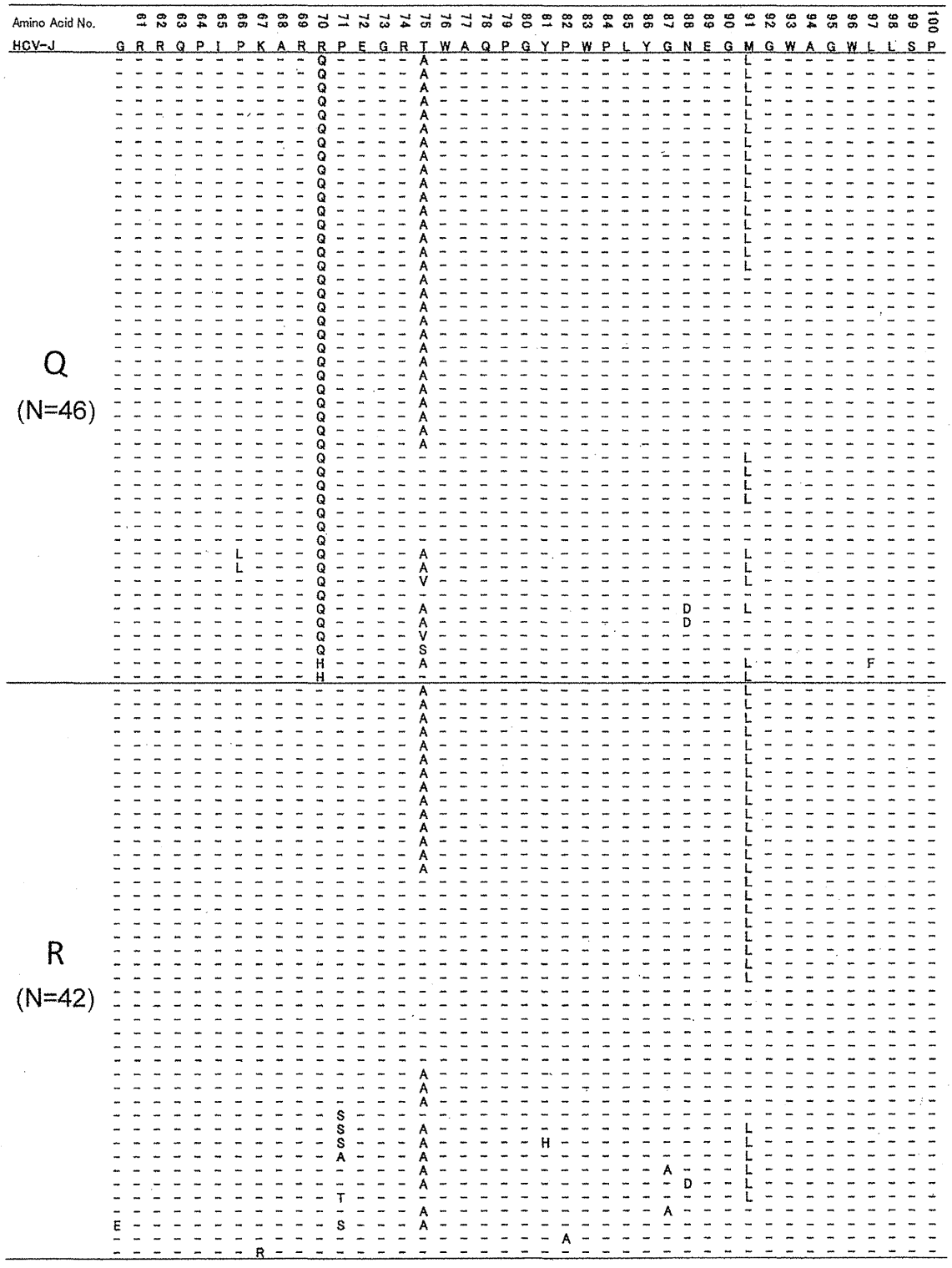

FIG 4 Dominant core amino acid haplotypic sequences dependent on the core aa 70 residues are given for all 79 patients. Two dominant sequences (a dominant core sequence in isolates with aa 70R and a dominant core sequence in isolates with aa 70non-R) were included for each of nine patients with high mixture rates (5% or more) of R and non-R at core aa 70, while one dominant sequence each was included for other patients. Therefore, a total of 88 haplotypic sequences are shown here. Since most amino acid mutations were observed in the limited core region, only that part of the core sequence is shown. The HCV-J isolate is used as the reference sequence for the core region.

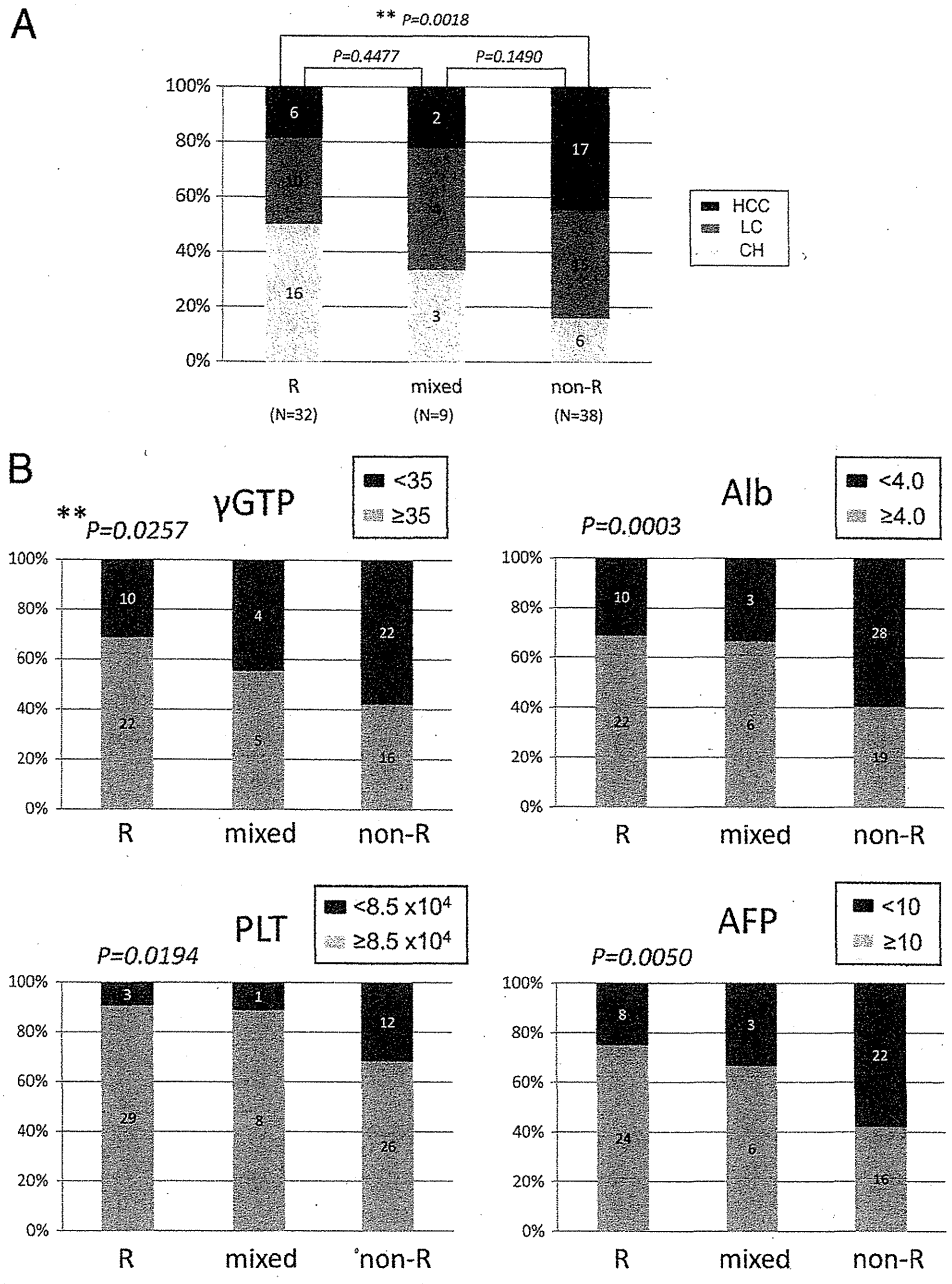

FIG 5 The advance of liver disease (A) and the levels of γ-GTP, albumin (Alb), platelets (PLT), and alpha-fetoprotein (AFP) (B) were investigated for correlation with the ratio of R to non-R at core aa 70. Results for R/R + non-R ratios of ≥95% (R), ≥5% and <95% (mixed), and <5% (non-R) are shown. **, Cochran-Armitage analysis.

because the presence of a quasispecies was expected at this position, considering reports by previous studies of its association with liver disease progression and the frequent observation of time-dependent changes (8, 9). R and non-R residues were mixed in 89.9% of the 79 patients examined in this study, indicating that the absence of a mixture was rare. Furthermore, the percentage of total isolates encoding non-R residues at this position showed a relationship with the advance of chronic liver disease, as shown in Fig. 2A and Table 3. Therefore, with regard to the relationship between the advance of liver disease and core aa 70, it may be accurate to say that a change in the ratio of amino acids at core aa 70, rather than mutation of core aa 70, was related to the advance of liver disease.

Because information for almost the entire core region was obtained from each patient, our next interest was to determine whether core aa 70 is associated with other viral regions. In other words, we sought to determine whether HCVs with core aa 70R and HCVs with core aa 70non-R are phylogenetically distinct variants. To clarify the issue, phylogenetic tree analysis using dominant sequences for the (almost-entire) core regions from all 79 patients was performed at first. This analysis disclosed, after the calculation of genetic distances, that core sequences with aa

70non-R were significantly more homogeneous than those with aa 70R, demonstrating that the hot spot core aa 70 residue is significantly associated with the remainder of the core sequence. Although the underlying mechanism is unclear, we speculated that the close correlation between core aa 70 residues and IL28B SNPs might have contributed to the result. That is, since endogenous IFN levels are known to be upregulated in patients with IL28B minor types (TG/GG) relative to those in patients with the IL28B major type (TT) in its natural state (28), it is possible that HCVs with core aa 70non-R, which are closely linked to IL28B TG/GG, are under strong antiviral pressure induced by IFN, resulting in the selection of more-homogeneous HCVs, which can survive in such an environment.

Considering this possibility, we proceeded to perform phylogenetic analyses of core sequences in single patients with high-percentage mixtures (5% or more) of R and non-R residues at core aa 70 by using deep-sequencing data, since the influence of endogenous IFNs was considered equal for all HCV isolates in a single patient. The deep-sequencing data showed that the genetic heterogeneity of core sequences in a single patient did not differ according to the core aa 70 residue but that core sequences formed distinct subgroups on the phylogenetic tree according to the core aa 70 residue (Fig. 3B), and this result was also proved by the calculation of genetic distances (Table 5). However, since no common haplotypic sequences specific to each residue at core aa 70 were found across the patients (Fig. 4), we cannot determine whether core aa 70R and aa 70non-R HCVs are phylogenetically distinct variants. It is possible that the result simply reflects a major evolutional event of core aa 70 mutations followed by derivative variants; however, extension of the investigation and analysis to viral regions beyond the core region might reveal such associations. However, due to the technical limitations of second-generation sequencers, deep-sequencing analysis of the long amplicon is difficult, and new technology is needed.

With regard to the mechanism underlying the relationship between the core protein and disease progression and hepatocarcinogenesis, a study using transgenic mice showed that the core protein induces HCC (29). Fat metabolism was accelerated in the liver, leading to inflammation, iron metabolism, oxidative stress, and insulin resistance, which were considered to be the carcinogenic factors (30–32). Clinically, mutation of the core and the concentration of γ-GTP in serum, a marker of steatosis, are related, and the relationship between IL28B SNP and liver steatosis or γ-GTP has been elucidated (33). In this study, moreover, we have confirmed the correlation between the core aa 70 mixture ratio, determined by deep-sequencing analysis, and clinical parameters reflecting disease progression, illustrating the significant association of core aa 70 with disease progression (Fig. 5A and B).

In conclusion, the quasispecies state of the core region was analyzed by deep sequencing. It was found that the status of the quasispecies was closely related to the advance of HCV-associated liver disease. In order to understand the mechanism of hepatocarcinogenesis, it is desirable to elucidate pathogenesis further by detailed examination of the quasispecies of the HCV core gene.

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