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Review Article

Liver transplantation for HIV/hepatitis C virus co-infected patients

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Since the introduction of antiretroviral therapy (ART) in the mid-1990s, AIDS-related death has been dramatically reduced, and hepatitis-C-virus (HCV)-related liver failure or hepatocellular carcinoma has currently become the leading cause of death in HIV/HCV co-infected patients. Liver transplantation may be one of the treatments of choices in such cases, but the indications for transplantation, perioperative management including both HIV and HCV treatments, immunosuppression and the prevention/treatment of infectious complications are all still topics of debate. With the improved understanding of the viral behaviors of both HIV and HCV and the development of novel strategies, especially to avoid drug interactions between ART and immunosuppression, liver transplantation has become a realistic treatment for HIV/HCV co-infected patients.

Key words: hepatitis C virus, HIV, liver transplantation

INTRODUCTION

IN JAPAN, IN the late 1980s, contaminated blood production of coagulation factor for hemophilia caused co-infection of HIV and hepatitis C virus (HCV). Actually, greater than 90% of HIV-infected patients have HCV as well.[1]

After antiretroviral therapy (ART) was introduced in the late 1990s, successful control of HIV was achieved in most cases and death due to AIDS was dramatically reduced, but HCV-related death due to liver failure or hepatocellular carcinoma became a serious problem, not only in Japan, but all over the world.[2–6] In such cases, liver transplantation (LT) is the only treatment option to achieve long-term survival, but several modifications of perioperative management are required. In this review, the outcome and the points of management of LT for HIV/HCV co-infected patients were reviewed.

REPORTED OUTCOME OF LT FOR HIV/HCV PATIENTS

THE REPORTED OUTCOMES of LT for HIV and HIV/HCV co-infected patients from Western countries after the introduction of ART are summarized in Table 1.[7–11] In general, most reports concluded that the results were worse than in the cases with HCV mono-infection, with a 3-year survival of approximately 60–70%. In Japan, the Tokyo group reported six cases of living donor liver transplantation (LDLT) between 2001 and 2004, of whom four died.[12] These unfavorable outcomes are likely related to the difficulties of determining the indications for LT and of perioperative management, including HIV/HCV treatment and the prevention and treatment of infectious complications. Terrault *et al.* reported that older donor age, combined kidney–liver transplantation, an anti-HCV positive donor and a body mass index of less than 21 kg/m^2 were independent predictors of graft loss.[10] After transplantation, several studies showed that acute cellular rejection was more frequent and severer in HIV/HCV co-infected patients than that in HCV mono-infected patients, possibly due to the difficulties in achieving optimal immunosuppression because of interactions between antiretroviral agents and immunosuppression.[10,11]

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Table 1 Outcome of liver transplantation for HIV/hepatitis C virus co-infection

Authors	Publication year	Country	*n*	Patient survival (%)		
				1 year	3 years	5 years
de Vera *et al.*[7]	2006	USA	27	67	56	33
Schreibman *et al.*[8]	2007	USA	15	73	73	–
Duclos-Vallee *et al.*[9]	2008	France	35	–	73	51
Terrault *et al.*[10]	2012	USA	89	76	60	–
Miro *et al.*[11]	2012	Spain	84	88	62	54

SPECIAL ISSUES REGARDING LT INDICATIONS FOR HIV/HCV CO-INFECTION

ART-related non-cirrhotic portal hypertension

IN HCV MONO-INFECTED patients, LT should be considered when the patients develop deteriorated liver function as indicated by a Child–Pugh classification of B or C. In HIV/HCV co-infected patients, liver failure due to HCV hepatitis was generally enhanced by ART-related hepatotoxicity, especially non-cirrhotic portal hypertension.[13–15] Accordingly, not only in cases with deteriorated liver function but also in class A cases, the patients can easily develop severe liver dysfunction suddenly,[16,17] so that all HIV/HCV co-infected patients should be carefully followed up so as not to miss the chance for LT. Also, Murillas *et al.* reported that Model for End-Stage Liver Disease (MELD) score is the best prognostic factor in HIV-infected patients,[18] so that HIV/HCV co-infected patients may be considered for LT before MELD score increase to achieve comparable results with HCV mono-infected patients. Several studies showed the aggressive fibrosis in HIV/HCV co-infected patients compared with HCV mono-infected patients,[19,20] but the mechanism of this aggressive fibrosis remains unclear. Recently, transient elastography or acoustic radiation force impulse imaging to check for liver stiffness has been introduced as an effective and non-invasive modality to determine patients' candidacy for LT.[21–23]

Count of CD4$^+$ T lymphocytes

Generally, the count of CD4$^+$ T lymphocytes has been required to be more than 200/μL to perform general elective surgeries in HIV-infected patients,[24] but in HIV/HCV co-infected patients, current studies show that a count of more than 100/μL is acceptable,[25,26] because patients generally have portal hypertension which can cause pancytopenia. In such patients, the ratio of CD4/CD8 is reported to be a feasible marker to predict postoperative complications including opportunistic infections. When the ratio is less than 0.15, the incidence of infectious complications is significantly higher.[27]

Preoperative infections

In regard to latent opportunistic infections that occur before LT, they are not absolute contraindications when they can be expected to be controlled.[28] Infections regarded as contraindications for LT included uncontrollable multidrug resistance HIV infection, chronic *Cryptosporidium enteritis*, progressive multifocal leukoencephalopathy and lymphoma.[29]

MANAGEMENT OF HIV/HCV IN LT

Management of HIV

THE NUMBER OF HIV RNA copies before LT is suggested as an independent risk factor of postoperative mortality, so that HIV should be controlled sufficiently before LT.[30] Accordingly, in the patients who are under consideration to receive LT, ART can be safely stopped before LT because HIV is generally well-controlled for a long period by ART. After LT, ART should be restarted as soon as possible because HIV RNA appears at 3–30 days after ART is stopped,[31] but the timing of restart of ART depends on the patient's condition, including liver function.[32] As long as the liver function has not fully recovered, or partial liver graft such as in LDLT has not sufficiently regenerated yet, ART cannot be started. Castells *et al.* reported in their case–control study that ART was started at a median of 8 days after LT (range, 4–28 days).[33] In principle, the ART administrated after LT should be the same as the pretransplant regimen, but the majority of ART drugs including protease inhibitor (PI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) have interactions with calcineurin inhibitors

(CNI) or mammalian target of rapamycin (mTOR),[34] so that the monitoring of blood levels of immunosuppression is extremely important to avoid infectious complications or rejection. Currently, a novel HIV-1 integrase inhibitor, raltegravir (RAL), is expected to be a feasible drug because it has no interactions with CNI, unlike other drugs.[35,36]

Management of HCV

The treatment strategy for HCV in HIV/HCV co-infected patients is the same as in HCV mono-infected patients. Combination therapy of pegylated interferon (PEG IFN) and ribavirin is the standard treatment both before and after LT. The timing of the induction therapy after LT is controversial. A Tokyo group proposed early induction as a preemptive therapy before patients develop hepatitis,[37] while several other reports showed favorable results when the treatment was administrated only after the development of hepatitis was confirmed by liver biopsy.[38,39] Theoretically, the treatment should be started as soon as possible, because in HIV/HCV co-infected patients, HCV recurrence may be accelerated in an immunocompromised state.[30,40] The novel protease inhibitor, telaprevir, is currently introduced as an effective drug to achieve sustained viral response of 70%, even in genotype 1b, with PEG IFN/ribavirin in a non-transplant setting,[41] but this drug is metabolized via cytochrome P450 as a substrate, as are CNI and various protease inhibitors of ART for HIV. Close monitoring of the CNI trough level should be performed, and although triple therapy with telaprevir/PEG IFN/ribavirin is currently reported to be effective to prevent HCV recurrence after LT in HCV mono-infected cases, special attention should be paid when this regimen is adapted in HIV/HCV co-infected patients.

IMMUNOSUPPRESSION

AS PREVIOUSLY MENTIONED, many factors including ART, anti-HCV treatment and an HIV-related immunocompromised state make post-LT immunosuppressive treatment difficult. Many ART drugs, both PI and NNRTI, cause instability in the blood concentration of CNI through the cytochrome P3A4 (CYP3A4)-related metabolism. Most PI cause the overconcentration of CNI by inhibiting CYP3A4, while most NNRTI cause decreased levels of CNI by stimulating CYP3A4.[29,42] As mentioned earlier, RAL is introduced as a key drug in LT in HIV positive patients, because the metabolism of this drug is not related to CYP450, so it does not affect the blood concentration of CNI. Several reports have demonstrated both the *in vitro* and *in vivo* effectiveness of rapamycin in reducing HIV replication,[43–45] and Di Benedetto *et al.* found that rapamycin monotherapy was significantly beneficial in long-term immunosuppression maintenance and HIV control after LT.[46] Mycophenolate mofetil is expected to be an effective immunosuppressive drug because of its efficacy in reducing HIV infection by both virological and immunological mechanisms.[47–49] Using these drugs, a more effective regimen of immunosuppression with ART may be established.

In regard to the steroid, several studies proposed that a steroid-free regimen can be safely applied and effective in LT for HCV cirrhosis. Also, in HIV/HCV co-infected patients, steroid-free protocol may be beneficial to prevent both HIV and HCV recurrence after LT.[50,51]

CONCLUSIONS

LIVER TRANSPLANTATION FOR HIV/HCV co-infected patients remains challenging, but with recent developments in perioperative management and novel drugs for both HIV and HCV, the results are likely to be improved.

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原　著

HIV/HCV 重複感染患者の肝障害病期診断における acoustic radiation force impulse（ARFI）elastography の有用性

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要旨：【背景】HIV コントロールの改善により HIV/HCV 重複感染者の死因として肝疾患の割合が増加している．【目的】重複感染者における acoustic radiation force impulse（ARFI）elastography による肝疾患進行度評価の有用性を明らかにする．【方法】肝実質硬度をせん断弾性波の速度（Vs）として定量化し，他の肝機能評価項目との相関を検討．【結果】Vs 値は血小板数，脾容積，ヒアルロン酸，IV 型コラーゲン，アシアロシンチ LHL15 値と有意な相関あり．【考察】ARFI は肝線維化・予備能評価に有用であり，HIV/HCV 重複感染者に対する非侵襲的で正確な肝疾患進行度評価に応用可能と考えられた．

索引用語：HIV/HCV 重複感染，肝移植，ARFI elastography，腹部超音波検査

はじめに

1990 年代後半の anti-retroviral therapy（ART）の登場によって human immunodeficiency virus（HIV）のコントロールは改善し，HIV 感染例の死亡数は減少するとともに死因に大きな変化が見られた．HIV 感染者における acquired immunodeficiency syndrome（AIDS）以外の死亡で最も多いのは肝疾患であり，その原因の多くは hepatitis C virus（HCV）感染症であった（Weber ら[1)]）．本邦においても平成 22 年度厚生労働省調査で，HIV/HCV 重複感染患者における死因の 1/3 は肝疾患であることが報告された[2)]．本邦における HIV 感染者の 19.2% が HCV に重複感染しており，その原因のほとんどが過去の HIV/HCV 混入血液製剤の投与であるが，血液製剤による HIV 感染者の HCV 抗体陽性率は 97% と極めて高い[3)]．このような薬害による HIV/HCV 重複感染者に対する的確な病期分類は，救済医療としての面からも今後その重要性が増すと考えられる．

重複感染者では単独感染者に比して線維化の進行が早いと報告されているが[4)]，過去の血液製剤の使用による重複感染者では血友病を有しているため，肝生検による線維化評価は困難である．近年，非侵襲的な肝線維化評価の方法として acoustic radiation force impulse（ARFI）elastography の有用性が報告されている[5)]．ARFI とは収束超音波パルスで組織に微細な変形をおこし，パルスが止んで組織が元の形に戻る際に体表に対して水平に発生するせん断弾性波の速度（velocity of shear wave；Vs）を測定し，組織の硬度を定量

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化したものである．今回われわれは，HIV/HCV重複感染者に対して，ARFI elastographyによる肝線維化評価の有用性を明らかにする目的で検討を行った．またわれわれは，重複感染者においては一般肝機能検査などが正常でも肝予備能が低下している症例が少なからず存在することを報告した（曽山ら[6]）．ARFIの測定結果と予備能の関連についても検討を行った．

I 対象と方法

2009年9月から2013年6月までに当院で精査を行ったHIV/HCV重複感染患者37名のうち，ARFI elastographyを施行したChild分類A症例23例を対象とした．37例はいずれも原疾患に血友病を有し，過去の血液製剤の使用によってHIV/HCV重複感染をきたした症例であり，全例男性，年齢の中央値は40歳（30～63歳）であった．肝機能の内訳はChild Aが34例，Bが1例，Cが2例であった．対照として同時期に当院消化器内科で加療を行ったHCV単独感染症例18例，および健常群として同時期に当院でグラフト採取術を施行した生体肝移植ドナー10例と比較を行った．まずARFIを用いて肝線維化をVsとして数値化し（右肋間より右葉のVs値を5回測定し平均値を用いた），重複感染群，HCV単独感染群，健常群でVs値の比較を行った．次にHIV/HCV重複感染群においてVs値（肝右葉・左葉それぞれでVs値を5回ずつ測定してそれぞれの平均値を算出し，左右の値の平均値を肝全体のVs値として用いた）とALT，総ビリルビン値，血小板数，CTでの肝形態，脾容積，ヒアルロン酸，IV型コラーゲン，ICG15分停滞率，アシアロシンチLHL15値との相関を検討した．脾容積はAquilion™，64列（東芝メディカルシステムズ，日本）を用いて動脈相，門脈相，平衡相の3相で造影CTを撮影し，平衡相の脾容積をSYNAPSE VINCENT（富士フイルムメディカル，日本）を用いてvolumetryを行い測定した．また肝形態の評価は当院の放射線科医師が読影し，正常肝の他に脂肪肝，慢性肝炎，肝硬変の4つに分類した．統計学的検定には，統計解析ソフトystat 2008（医学図書出版，東京）を用い，2群間比較にはMann-Whitneyの順位和検定を，相関についてはSpearmanの順位相関を行った．

II 結 果

Vs値（以下中央値と範囲）はHIV/HCV重複感染群で1.27（0.98～2.61）m/s，HCV単独感染群で1.27（0.85～3.00）m/s，健常群で1.08（0.98～1.33）m/sであり，重複感染群は健常群に比べて有意に高値であった（$p=0.010$）が，重複感染群とHCV単独感染群，およびHCV単独感染群と健常群では有意差を認めなかった（それぞれ$p=0.436$，$p=0.059$，Figure 1）．またARFI施行時の年齢については，重複感染群が46（31～63）歳に対して単独感染群が61（33～76）歳と，重複感染群は単独感染群に比べて有意に若年であった（$p=0.008$，Figure 1）．

重複感染群におけるVs値と他の肝機能検査の比較では，ALT（$p=0.358$）や総ビリルビン値（$p=0.949$）では両群に相関を認めなかったが，血小板数（$r=0.737$，$p<0.001$），脾容積（$r=0.592$，$p=0.006$），ヒアルロン酸（$r=0.637$，$p=0.003$），IV型コラーゲン（$r=0.569$，$p=0.009$）はVs値と有意な相関を認めた（Figure 2）．またCTで正常肝を示したものは23例中6例のみで，その他の内訳は脂肪肝1例，慢性肝炎8例，肝硬変8例であった．CTによる形態評価とVs値の相関については，正常肝6例で1.24（1.11～2.12）m/s，その他の17例では1.87（1.14～3.04）m/sであり，両群に有意差を認めなかった（$p=0.058$）．肝予備能評価項目との比較検討では，ICG15分停滞率（$p=0.054$）とは相関を認めなかったものの，アシアロシンチLHL15（$r=0.503$，$p=0.024$）とは有意な相関を認めた（Figure 3）．

III 考 察

HIV/HCV重複感染者のChild A症例においては，健常者と比較してVs値が有意に高値であり，ARFI elastographyの線維化測定は正しく行われていると考えられた．またVs値は，肝線維化のマーカーとして知られるヒアルロン酸やIV型コラーゲン，門脈圧亢進症の所見である血小板数，脾容積と相関を認めた．今回の検討では血友病のため肝生検を行っておらず，組織学的な線維

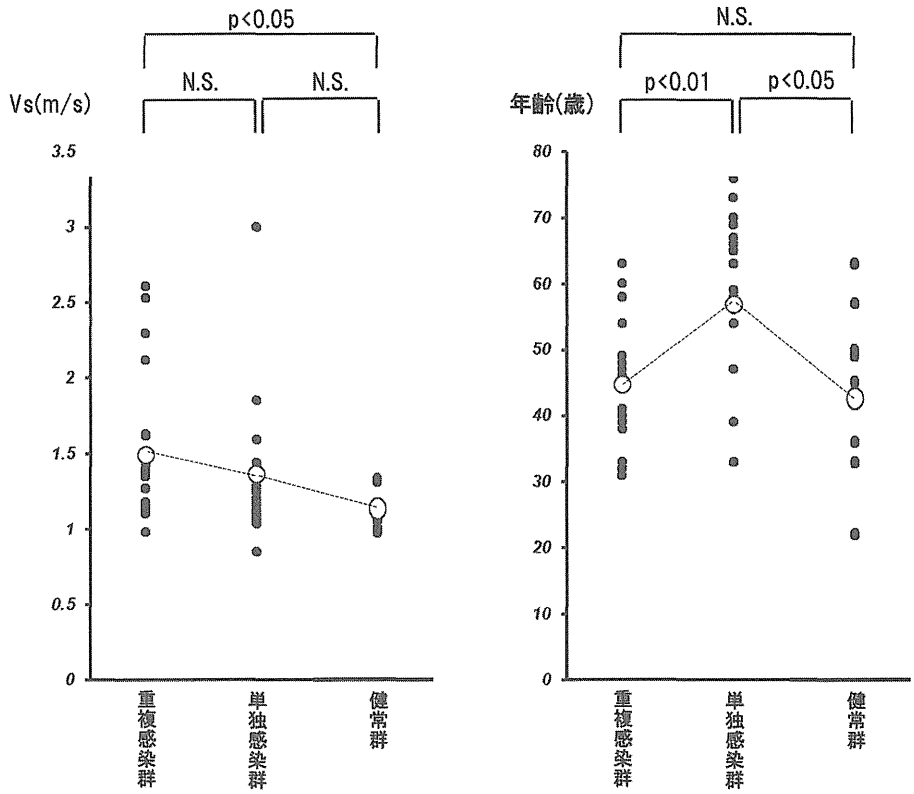

Figure 1. Comparison of Vs and age among HIV/HCV co-infected patients, HCV mono-infected patients, and healthy control (living donor liver transplantation (LDLT) donor).

化の評価はできていないが，HIV/HCV 重複感染者では Child A 症例であっても肝線維化が進行している症例を含んでいることを反映していると思われた．重複感染群と HCV 単独感染群との比較では Vs 値に有意差を認めなかったが，測定時の年齢については，重複感染群の方が有意に若年であった．視点を変えれば，重複感染においては若年者でも既に単独感染の高齢者と同等の線維化をきたしているということになる．重複感染者においては肝線維化の進行が HCV 単独感染群より早いことが諸家から報告されている[4]ことを念頭に，若年期から適切な治療のタイミングを逸しないように，綿密なフォローアップを行うことが重要である．

重複感染者においては HIV/HCV の相互作用や ART による薬剤性肝障害によって肝線維化の進行が早い事例が存在することに加えて，肝実質障害に比して門脈圧亢進症の進行が早い非硬変性門脈圧亢進症の病態を呈することもあり[7][8]，一般肝機能検査のみでは病勢を正確に評価できないケースを多数認める．今回の検討では Vs 値は ALT やビリルビン値などの一般肝機能検査とは相関を示さず，また CT で正常肝であった症例と異常を認めた症例間の Vs 値に有意差は認めなかった．ARFI により Vs 値を測定することは従来の一般肝機能検査とは違った角度から肝機能を評価することとなり，より正確な病期診断につながる可能性が示唆された．

今回の検討で興味深いのは，ARFI elastography の結果が，肝予備能評価と相関していたことである．重複感染者は現在全国各地でフォローアップされているが，肝臓病専門施設や地域中核病院から遠方に居住する患者では，定期的な肝予備能評価が行われていないことも少なくない．さ

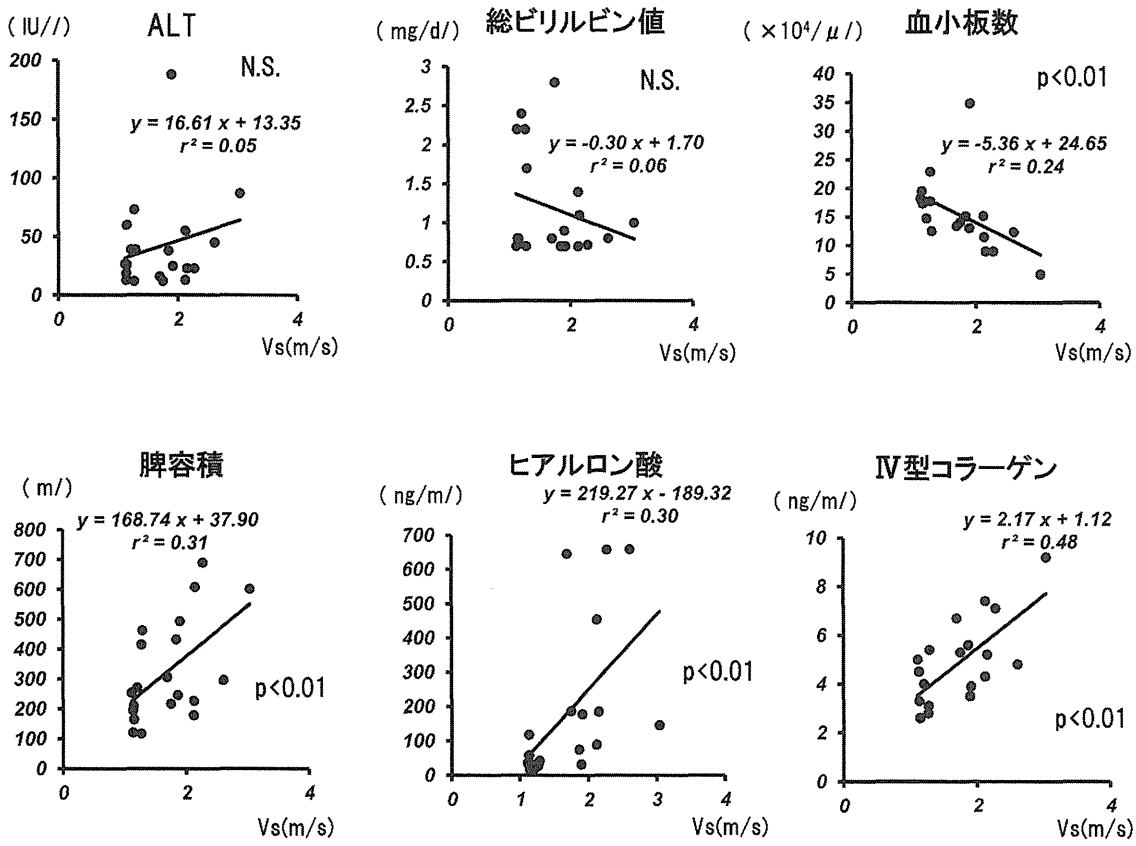

Figure 2. Correlation between Vs and other liver function (ALT, total bilirubin, and platelet counts), splenic volume, hyaluronic acid, type Ⅳ collagen.

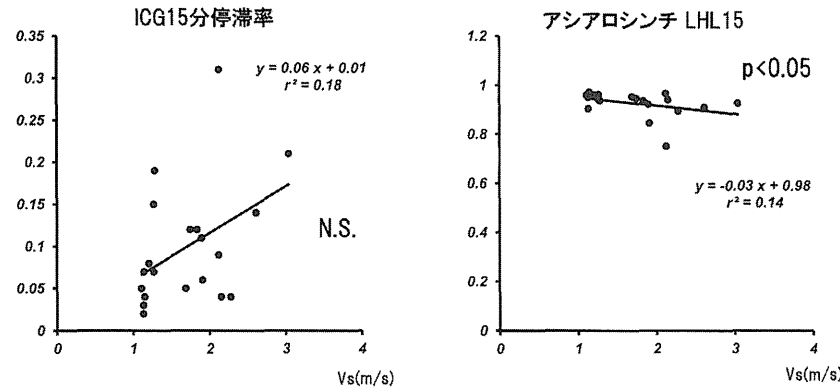

Figure 3. Correlation between Vs and hepatic functional reserve (indocyanine green retention rate and LHL15 in 99mTc-GSA scintigraphy).

らに本邦における重複感染の原因の大半は血友病に対する血液製剤使用にあり，凝固異常のため肝生検による病期診断が困難であるという点が，重複感染者の病期評価を困難にしている一因と思わ

れる．超音波検査はシンチグラムなどに比べて簡便で特別な装置を必要とせず，肝疾患専門施設以外でも導入しやすいと思われる．また凝固能の異常があっても繰り返し施行することが可能であり，病状の進行を経時的に評価することができる．このように従来の肝線維化・肝予備能の指標と有意な相関を示し，かつ非侵襲的なARFI elastographyは有用な診断ツールと考えられる．

前述の如く，HIV/HCV重複感染者における肝線維化の進行は多因子に影響される．ARFIにより肝線維化の程度を数値化し，その経時的測定値と，各種臨床データや服薬歴などとの相関を明らかにすることで，重複感染者における線維化進行のメカニズム解明につながる可能性がある．ひいては個々の症例が有する危険因子から，肝疾患の正確な予後予測へとつながることが期待される．

HIV/HCV重複感染者におけるelastographyを用いた肝線維化評価としては，transient elastography（FibroScan®，Echosens，フランス）の測定結果と組織学的な肝線維化進行度との有意な相関が報告されている[9)10)]．FibroScanもARFIと同様にせん断波の速度を測定することにより肝硬度を評価する装置であるが[11)]，FibroScanは低周波弾性波を用いて体表と垂直方向の弾性波伝搬速度を測定するため，腹水貯留例や高度肥満例においては測定が困難とされている[12)]．またARFIではBモードも可能であり，ルーチンの観察を行った後に肝線維化を測定するといった使用法も可能であるという利点がある．ARFIとFibroScanの精度については，ウイルス性肝炎における検討ではほぼ同等とする報告が多い[5)13)]．HIV/HCV重複感染者におけるARFIと組織学的線維化の相関についてはいまだ報告はなく，今後の検討が必要と思われる．

近年，HIV/HCV重複感染者に対する肝移植の成績が欧米を中心に報告されており，その成績は3年生存率が60%程度であった[14)]．これはHCV単独感染よりやや不良であるが徐々に改善が見られている．近年ではRaltegravirのようにTacrolimusとの相互作用を認めない抗HIV薬も登場しており，今後移植後の免疫抑制剤の調整が容易となることでさらなる成績の改善が期待される[15)16)]．

結　語

ARFI elastographyは，HIV/HCV重複感染者の肝線維化や肝予備能評価のツールとして，肝疾患病期診断に有用であり，肝移植も含めた適切な治療の選択の判断材料となると思われた．

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本論文内容に関連する著者の利益相反
：なし

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Acoustic radiation force impulse elastography for liver disease staging in human immunodeficiency virus and hepatitis C virus co-infection

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Background : Survival of human immunodeficiency virus (HIV)-infected patients has improved due to the widespread use of anti-retroviral therapy. However, mortality has increased when HIV-infected patients are co-infected with hepatitis C virus (HCV), and the liver disease in such patients is rapidly progressive compared with that in HCV monoinfected patients. Therefore, accurate staging of the liver disease is critical when determining appropriate treatment. Aim : To clarify the efficacy of acoustic radiation force impulse (ARFI) elastography for the evaluation of liver fibrosis and hepatic functional reserve in HIV/HCV co-infected patients. Methods : The correlation of shear wave velocity (Vs), measured by ARFI elastography, with liver fibrosis or hepatic functional reserve was analyzed. Results : Vs was significantly correlated with platelet count, splenic volume, hyaluronic acid, type IV collagen, and LHL15 (receptor index : uptake ratio of the liver to the liver plus heart at 15min) in 99mTc-GSA (technetium-99m-diethylenetriaminepentaacetic acid-galactosyl human serum albumin) scintigraphy. Conclusion : ARFI elastography was useful for the staging of liver disease in HIV/HCV co-infected patients and it facilitated minimally invasive and accessible evaluation of fibrosis and functional reserve.

DNA methylation at hepatitis B viral integrants is associated with methylation at flanking human genomic sequences

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Integration of DNA viruses into the human genome plays an important role in various types of tumors, including hepatitis B virus (HBV)–related hepatocellular carcinoma. However, the molecular details and clinical impact of HBV integration on either human or HBV epigenomes are unknown. Here, we show that methylation of the integrated HBV DNA is related to the methylation status of the flanking human genome. We developed a next-generation sequencing-based method for structural methylation analysis of integrated viral genomes (denoted G-NaVI). This method is a novel approach that enables enrichment of viral fragments for sequencing using unique baits based on the sequence of the HBV genome. We detected integrated HBV sequences in the genome of the PLC/PRF/5 cell line and found variable levels of methylation within the integrated HBV genomes. Allele-specific methylation analysis revealed that the HBV genome often became significantly methylated when integrated into highly methylated host sites. After integration into unmethylated human genome regions such as promoters, however, the HBV DNA remains unmethylated and may eventually play an important role in tumorigenesis. The observed dynamic changes in DNA methylation of the host and viral genomes may functionally affect the biological behavior of HBV. These findings may impact public health given that millions of people worldwide are carriers of HBV. We also believe our assay will be a powerful tool to increase our understanding of the various types of DNA virus-associated tumorigenesis.

[Supplemental material is available for this article.]

Hepatitis B virus (HBV) infects more than two billion people worldwide, and 400 million chronically infected individuals are at high risk of developing active hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Gatza et al. 2005; Lupberger and Hildt 2007). HBV carriers with chronic liver disease are at a 100-fold greater risk of developing HCC, which is the third leading cause of cancer-related death worldwide. The HBV genome is integrated into the host genome in 90% of patients with HCC (HBV-HCC) (Gatza et al. 2005; Lupberger and Hildt 2007). HBV-HCCs have been analyzed by comprehensive genome sequencing and high-resolution genome mapping (Kan et al. 2013; Li and Mao 2013; Nakagawa and Shibata 2013). Moreover, the recent deep sequencing of HBV DNA in patients with HCC revealed increased integration events, structural alterations, and sequence variations (Ding et al. 2012; Fujimoto et al. 2012; Jiang et al. 2012; Sung et al. 2012; Toh et al. 2013). A recent study identified a viral-human chimeric fusion transcript, HBx-LINE1, that functions like a long noncoding RNA to promote HCC (Lau et al. 2014). However, the molecular details and clinical impact of HBV integration on the epigenomes of human cells and HBV remain to be defined.

Methylation of exogenous DNA (including viral DNA) that is integrated into the human genome has been studied over the past decade (Doerfler et al. 2001). Within the human genome, cytosine methylation in CpG dinucleotides (CpG sites), which cluster into islands associated with transcriptional promoters, is an important mechanism for regulating gene expression. Additionally, host cells use methylation as a defense mechanism against foreign agents (e.g., viral DNA) (Doerfler 2008; Doerfler et al. 2001). DNA methylation suppresses the expression of viral genes and other deleterious elements incorporated into the host genome over time. Establishment of de novo patterns of DNA methylation is char-

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acterized by the gradual spread of methylation (Orend et al. 1991). Another attractive possibility is that DNA methylation camouflages the virus from the immune system (Tao and Robertson 2003; Hilleman 2004), resulting in a DNA methylation–related blockade of viral antigen presentation that allows the virus to escape immune control (Fernandez et al. 2009).

The DNA methylome of HBV in human cells may undergo dynamic changes at different stages of disease (Fernandez et al. 2009). For example, DNA methylation at the *HBVgp2* locus, which codes for the S viral proteins, reportedly increases during the progression from asymptomatic lesions to benign lesions, to premalignant disease and malignant tumors. However, because of the significant deletions of the integrated HBV genome detected in this previous study (Fernandez et al. 2009), the DNA methylome of HBV needs to be further characterized. Moreover, the molecular mechanisms involved and the clinical impact of the integration of HBV on the human and HBV epigenomes are unknown. To address these issues, we developed a next-generation sequencing (NGS)–based method for methylation analysis of integrated viral genomes (denoted G-NaVI) and applied this method to the integrative genomic and epigenomic analysis of human hepatoma cell lines and tissues with integrated HBV genomes.

Results

DNA methylation levels in PLC/PRF/5 cells and cancerous tissues obtained from HBV-HCC patients

Methylated CpG island (CGI) amplification (MCA) coupled with microarray (MCAM) analysis (Toyota et al. 1999; Oishi et al. 2012) was performed to detect methylated genes in the human PLC/PRF/5 cell line and in six paired specimens of primary HBV-HCC and adjacent tissues. Compared with the DNA methylation of CGIs in the healthy peripheral blood leukocytes of volunteers or the noncancerous tissues, levels of DNA methylation were not remarkable in the PLC/PRF/5 cells and the cancerous tissues obtained from HBV-HCC patients (Supplemental Fig. 1). These results were confirmed by bisulfite pyrosequencing of candidate tumor-related genes.

DNA methylation of CGIs of *HBx*

We then focused on epigenetic changes in the viral genome. Based on the hidden Markov models for sequence analysis performed on the CpG plugin of bioinformatics software Geneious 5.5.8 (see Methods section), a CpG island was found in only the promoter region of the *HBx* gene in the HBV genome (Fig. 1A; Supplemental Fig. 2; Durbin et al. 1998; Kearse et al. 2012). Host signal transduction pathways and gene expression are disrupted by the expression of *trans*-activating factors derived from the HBV genome, such as the HBx protein and PreS2 activators (Gatza et al. 2005; Lupberger and Hildt 2007). Moreover, transgenic mice expressing high levels of HBx in the liver develop HCC (Kim et al.1991; Koike et al. 1994). The DNA methylation levels of the CGIs of *HBx* were analyzed in 10 HBV-HCC samples and 10 adjacent samples, as well as samples of PLC/PRF/5 cells by bisulfite pyrosequencing (Fig. 1A; Supplemental Fig. 2). We performed advanced methylation quantification in long sequence runs by pyrosequencing on PyroMark Q24 Advanced and PyroMark Q24 instruments. Methylation levels of *HBx* varied across samples (Fig. 1B,C) and were generally lower in HCC tissues than in the adjacent tissues (Fig. 1B). This finding is consistent with a previous report that most HBV genomes, although globally methylated to a greater extent in malignant samples than in premalignant lesions, retain *HBx* in an unmethylated state (Fernandez et al. 2009). Because the pyrosequencing results represent the genome-wide average of DNA methylation levels at the particular CpG site, the results could be affected by the HBV integration site. Therefore, genome-wide methylation analysis of the integrated HBV sequence is necessary in relation to the methylation state of the adjacent human genome. We did not detect an association between *HBx* methylation levels and those of the LINE1 and *Alu*Yb8 repeats (Fig. 1B).

Fluorescence in situ hybridization (FISH) and *Alu* PCR analyses of HBV integration

We developed a FISH technique for detecting HBV DNA in the genome of PLC/PRF/5 cells (Supplemental Figs. 3, 4). Twelve specific primer pairs (FISH probes 1–12) were designed based on the HBV sequences integrated into the genome of PLC/PRF/5 cells; amplification from all primer pairs was confirmed (Supplemental Fig. 4A). These results suggest full-length or partial HBV sequences that are covered by the 12 primer pairs were integrated into the genome of the PLC/PRF/5 cells. The FISH probes were labeled with digoxigenin, and FISH was performed using Carnoy-fixed chromosomal and nuclear specimens. Multiple HBV fluorescent signals (green) were detected in the nuclei (Supplemental Fig. 4B) using probes for *HBx* and its CGI sequences (probes 5 and 6), but not with probes 1–4 or 7–12 (Supplemental Fig. 4C–E). *Alu*-PCR identified one *HBx* integration site in PLC/PRF/5 (Supplemental Fig. 5). The integrated *HBx* sequence was 213 bp and included a promoter region. The *HBx* gene body was located only 13 bases (ATG GCT GCT AGG T) from the transcription start site and was integrated into a noncoding region of the host genome. There were 200 bases of viral DNA sequence upstream of the *HBx* transcription start site. According to the human genome reference sequence (GRCh38) published by the Genome Reference Consortium, this integration site was identified as a noncoding region of host Chromosome 5 1,350,106–1,350,478 that is near the telomerase reverse transcriptase (*TERT*) gene (Supplemental Fig. 5).

NGS analysis of HBV DNA integration site sequences

We developed an NGS analysis technique for sequencing the HBV DNA integration sites (Supplemental Fig. 6A). For efficient genome analysis, we synthesized 12,391 custom baits based on the sequences of the HBV genotypes A to J and on those sequences present in the HBV-transformed PLC/PRF/5 cells that were not related to the human genome sequence (Supplemental Fig. 6B). The average read length was 333.14 bp with a modal length of ~500 bp (Supplemental Fig. 6C). The average read quality was 31.91, corresponding to >99.9% accuracy. We did not detect a common HBV integration site (Fig. 2). The integration sites in the PLC/PRF/5 genome included intergenic (39%), intronic (39%), promoter (8%), and divergent promoter (15%) regions but not exonic (0%) sequences (Fig. 2). HepG2.2.15 cells, which stably express and replicate HBV in a culture system, are derived from the human hepatoblastoma cell line HepG2 (Sells et al. 1987). In the HepG2.2.15 genome, the integration sites included intergenic (29%), intronic (57%), and other (14%) regions but not promoter (0%), divergent promoter (0%), or exonic (0%) sequences (Fig. 2).

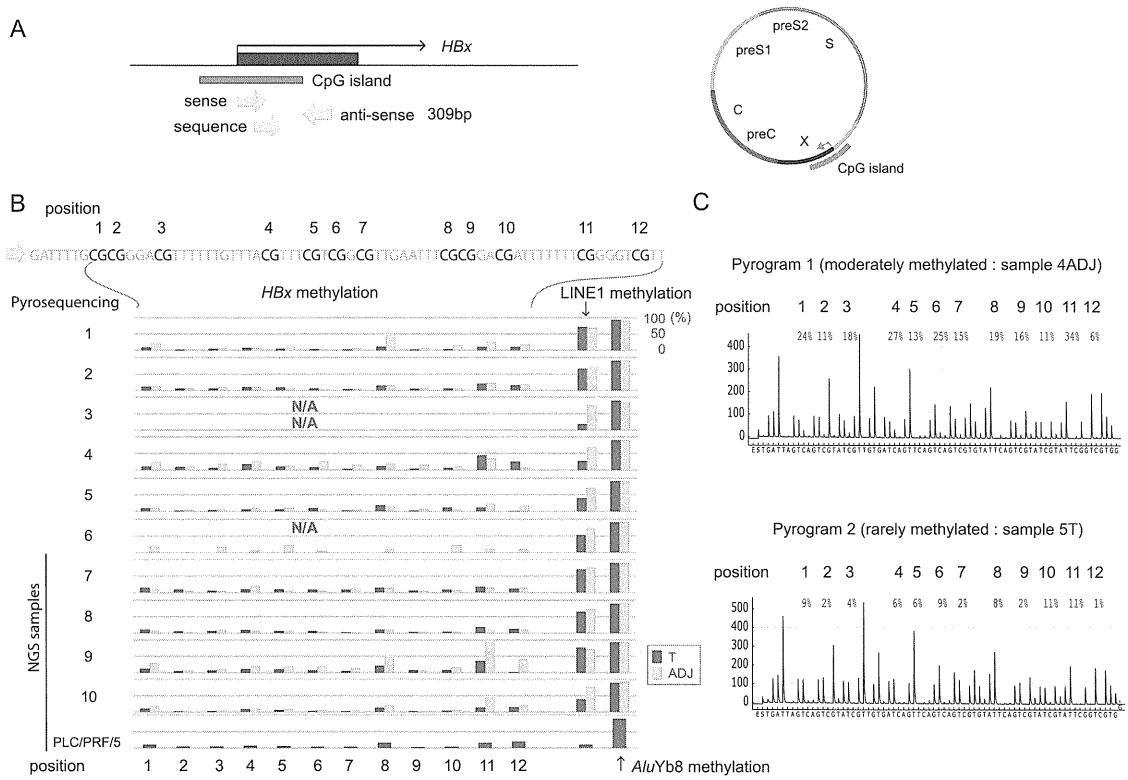

Figure 1. Methylation analysis of the CGI of the *HBx* gene. (*A*) Schema of the CGI of the *HBx* gene. Three arrows show the pyrosequencing primers used for the methylation analysis. (*B*) DNA methylation levels of the CpGs of the *HBx* gene, LINE1, and *AluYb8* in 10 paired HBV-HCC and adjacent nontumor tissue samples and PLC/PRF/5 DNA were analyzed using bisulfite pyrosequencing. Methylation levels of *HBx* varied across samples and were generally lower in HCC tissues than in the adjacent nontumor tissues. An association between *HBx* methylation levels and those of the LINE1 and *AluYb8* repeats was not observed. N/A, could not be analyzed. DNAs from four paired HBV-HCC and adjacent nontumor tissue samples (sample nos. 7–10), PLC/PRF5, and HepG2.2.15 were further analyzed using the NGS (G-NaVI method). (C) Representative pyrograms showing DNA methylation levels of the CpGs of the *HBx* gene. Methylation levels at 12 CpG sites of the *HBx* gene in adjacent nontumor tissue (sample no. 4ADJ) and tumor tissue (sample no. 5T) are shown.

DNA methylation of the integrated HBV genome as well as the adjacent human genome in cell lines

DNA methylation of the integrated HBV genome, as well as the adjacent human genome, was analyzed by bisulfite pyrosequencing. We detected varying levels of methylation of the HBV sequences integrated into the genome of PLC/PRF/5 cells (Fig. 3; Supplemental Fig. 7). Our data suggest DNA methylation in the integrated HBV genome is related to the methylation status of the integration sites within the human genome. We further characterized the methylation status of the HBV genome and human genome by allele-specific DNA methylation analysis (Fig. 3A), which revealed that the HBV genome often showed significant methylation when integrated into highly methylated sites in the human genome; however, the HBV genome remained largely unmethylated when integrated into unmethylated regions such as promoters (Fig. 3B). Integration of the HBV genome did not affect the methylation status of the human genome, including the promoter regions of the *TERT* and *SNX15* genes. Methylation of HBV DNA integrated into HepG2.2.15 cells transformed with HBV DNA (using a head-to-tail dimer) was further analyzed by bisulfite pyrosequencing, which revealed that the HBV genome generally showed significant methylation when integrated into highly methylated regions of the human genome; however, the HBV genome remains largely unmethylated when integrated into unmethylated regions (Fig. 3A).

DNA methylation levels in orthologous loci

We examined methylation levels of orthologous loci in HepG2.2.15 cells and in peripheral blood lymphocytes (PBLs) of a healthy volunteer and compared them to the methylation levels at the same (empty) target sites of PLC/PRF/5 cells. Methylation levels of orthologous loci in HepG2.2.15 cells and PBLs were generally similar to those of PLC/PRF/5 cells (Fig. 3B). Similarly, we examined methylation levels of orthologous loci in PLC/PRF/5 cells and in PBLs of a healthy volunteer and compared them to the methylation levels at the same (empty) target sites of HepG2.2.15 cells. Methylation levels of orthologous loci in PLC/PRF/5 cells and PBLs were also generally similar to those of HepG2.2.15 cells (Fig. 3B).

DNA methylation of the integrated HBV genome and the adjacent human genome in HCC tissues

To determine whether our results are relevant to human tumors, we used bisulfite pyrosequencing to investigate the methylation status of the HBV and human genomes in surgical specimen pairs of HCC and adjacent nontumor tissues. We detected no common HBV integration site (Fig. 4; Supplemental Fig. 8). Recurrent HBV integration into the *SLC6A13* gene was observed in cancerous tissues. Integration sites were rarely detected in exonic regions of the DNA from HBV-HCC samples (Fig. 4; Supplemental Fig. 8). Similar to the results obtained from the PLC/PRF/5 and HepG2.2.15 cells, our analysis revealed that the HBV genome became significantly

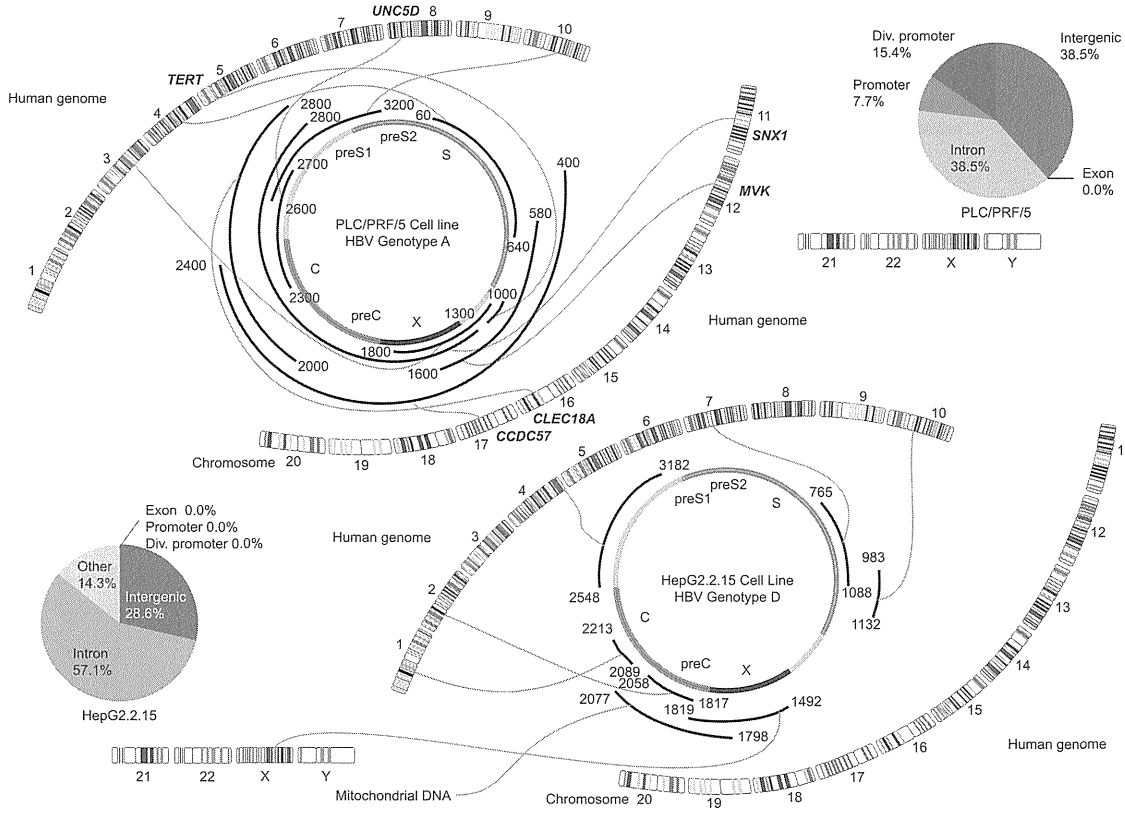

Figure 2. Distribution of the integration sites in the HBV genome and human chromosomes represented by Circos plots of the PLC/PRF/5 genome and the HepG2.2.15 genome. HBV DNA integration was analyzed using the G-NaVI method in the genome of PLC/PRF/5 cells and HepG2.2.15 cells. A common HBV integration site was not detected. Integration sites were not detected in exonic regions of the DNA from cell lines (Venn diagrams). The HBV genes (*PreC, Precore; C, Core; PreS, Presurface; S, Surface; X, X*) and the 24 human chromosomes are shown.

methylated when integrated into highly methylated human genome regions but not when integrated into unmethylated human genome regions (Fig. 4).

Correlation between the methylation pattern of the integrated HBV DNA and the human genome

DNA fragments, including 200 bp of the HBV DNA and 200 bp of the human genome around the boundary, were analyzed for average methylation, GC content, and repetitive sequences. A statistically significant correlation was observed between the average methylation of the HBV DNA and that of the human genome in cell lines and clinical samples (Fig. 5A–C; Supplemental Table 2). In contrast, average methylation did not correlate with GC content or repetitive sequences in the human and viral genome (Fig. 5D,E; Supplemental Table 2).

Using Bander software, we analyzed the chromatin structure at the integrated HBV site in PLC/PRF/5 and HepG2.2.15. Open chromatin and heterochromatin were observed more frequently at the integrated HBV in PLC/PRF/5 and HepG2.2.15, respectively (Supplemental Table 3). The difference may reflect the fact that PLC/PRF/5 is a naturally derived HBV-positive cell line and HepG2.2.15 is an HBV DNA-transfected cell line.

Discussion

We developed an NGS-based method for structural methylation analysis of integrated viral genomes. This method is a novel approach that enables the enrichment of viral fragments for sequencing using unique baits based only on the sequence of the HBV genome. We detected all regions of the human genome that harbored integrated HBV genomes without conducting unnecessary sequencing of regions where the HBV genome was not integrated. Because this technique only requires sequencing a small region of DNA around the integrated HBV sequences, a sufficient number of sequence reads can be acquired.

Methylation of viral DNA in infected cells may alter the expression patterns of viral genes related to infection and transformation (Burgers et al. 2007; Fernandez et al. 2009) and may clarify why certain infections are either cleared or persist with or without progression to precancer (Mirabello et al. 2012). To the best of our knowledge, we have, for the first time, established that the de novo patterns of DNA methylation in the integrated HBV genome are related to the methylation status of the integration sites within the human genome. A statistically significant correlation between the average methylation of the HBV DNA and that of the human genome in cell lines and clinical samples has greatly substantiated our findings. It is possible that the HBV genome becomes inactivated by methylation, when it is integrated into highly methylated host sites; therefore, HBV methylation may not contribute to tumor development. However, after integration into unmethylated human genome regions such as promoters, the HBV DNA remains unmethylated and may eventually play an important role in tumorigenesis (Fig. 6). Because multiple HBV integration sites were present in each of the analyzed samples, there remains the possibility of an asso-

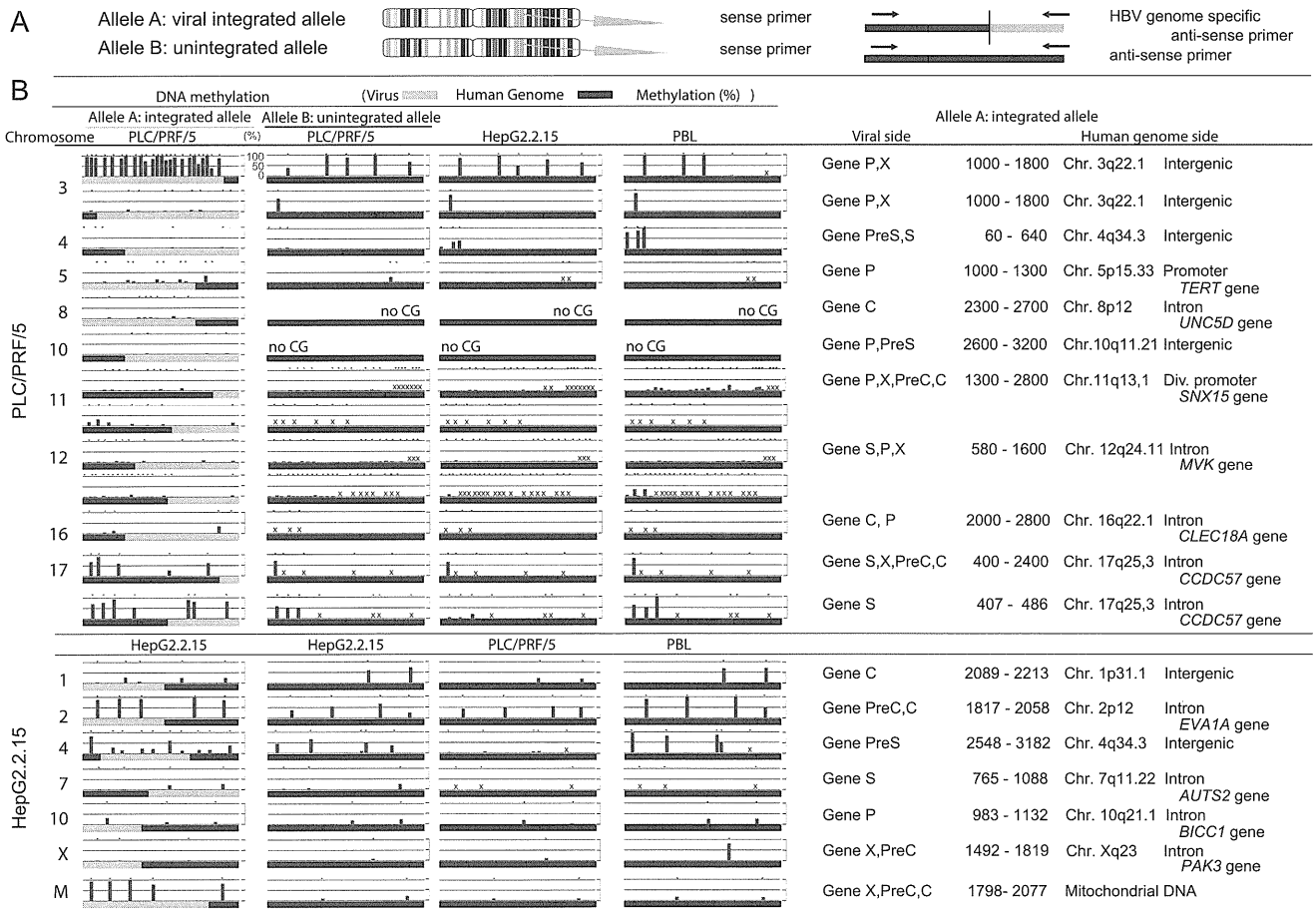

Figure 3. Allele-specific methylation analysis of the PLC/PRF/5 genome and the HepG2.2.15 genome. (*A*) A schema of allele-specific methylation analysis. (*B*) The methylation levels of the HBV and human genomes for the integrated and unintegrated alleles. Detailed results of the HBV integrants (*PreC, Precore; C, Core; PreS, Presurface; S, Surface; X, X*) and flanking host genomes (position, chromosome, location of the genome, and gene names) are shown. DNA methylation of the integrated HBV genome as well as the flanking human genome was examined by allele-specific DNA methylation analysis using bisulfite pyrosequencing. The HBV genome often showed significant methylation when integrated into highly methylated sites in the human genome; however, the HBV genome remained largely unmethylated when integrated into unmethylated regions. Methylation levels of orthologous loci in HepG2.2.15 cells and in PBLs of a healthy volunteer were examined and compared to the methylation levels at the same (empty) target sites of PLC/PRF/5 cells. Methylation levels of orthologous loci in HepG2.2.15 cells and PBLs were generally similar to those of PLC/PRF/5 cells. Similarly, methylation levels of orthologous loci in PLC/PRF/5 cells and in PBLs of a healthy volunteer were examined and compared to the methylation levels at the same (empty) target sites of HepG2.2.15 cells. Methylation levels of orthologous loci in PLC/PRF/5 cells and PBLs were generally similar to those of HepG2.2.15 cells. (×) The desired quantitative methylation levels were not obtained because of technical difficulties with the sequences that were being analyzed.

ciation between methylation and viral transcript levels. The biological impact of methylation on viral transcript levels or viral function, induced by viral insertions, also needs to be further addressed.

Methylation levels of orthologous loci in other samples at the same (empty) target sites of PLC/PRF/5 were generally similar to those of PLC/PRF/5. Similar results were observed in HepG2.2.15. These data suggest that a "before and after" relationship exists between methylation levels at preexisting target sites and those within viral insertions. At the same time, we cannot rule out the possibility that the integration of the virus subsequently affects the methylation established at the flanking target site, perhaps by acting in trans on the empty target site-containing allele. Therefore, this issue needs to be further addressed.

Differences in the integrated viral sequences could have a direct impact on the amount of cytosine methylation observed. In cases where the integration site is a highly active promoter, comparisons of methylation statuses may not be informative. Additional studies, using a large number of samples, are needed to address this issue.

Our results are notable because other studies have detected a statistically significant enrichment of HBV integration into regulatory regions, particularly promoters, in tumors (Sung et al. 2012; Toh et al. 2013); this observation may be explained by the relatively open chromatin structure of promoter regions. Average methylation did not correlate with GC content or repetitive sequences in the human and viral genomes. The relationship between methylation of HBV sequences and chromatin structure remains to be clarified because of the limitation of the Bander software used in this study. Although the mechanism needs clarification, the significant enrichment of HBV integration into regulatory regions would favor integrated HBV nonmethylation and lead to tumorigenesis. Alternatively, while the integration of HBV into the host genome may be random, HBV integration into regulatory regions is positively selected during tumorigenesis (Toh et al. 2013).

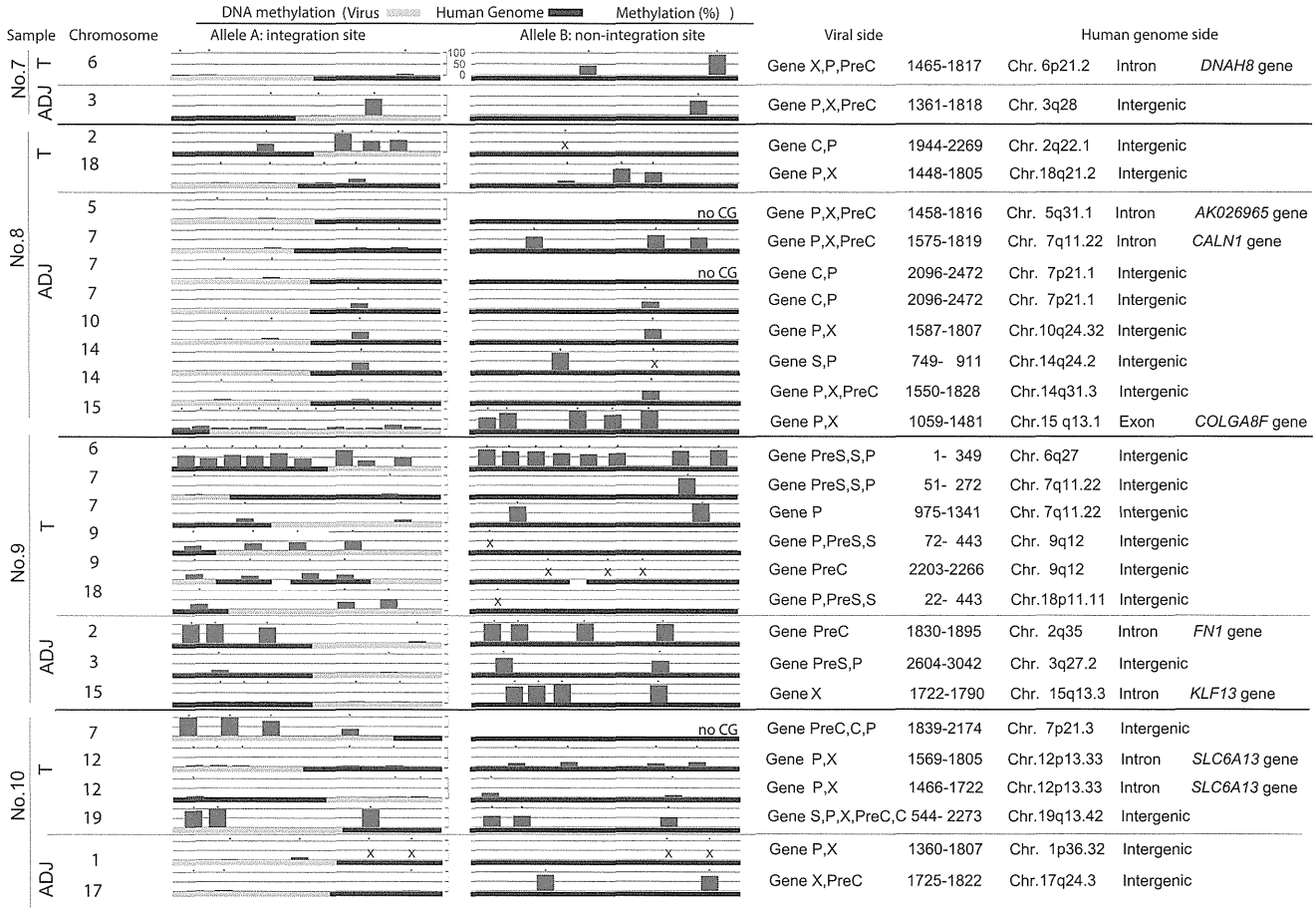

Figure 4. Allele-specific methylation analysis of the tumor (*T*) and adjacent nontumor (ADJ) sample genomes. The methylation levels of the HBV and human genomes for the integrated and unintegrated alleles in four paired tumor and adjacent nontumor samples (sample nos. 7–10) are shown. Detailed results of the HBV integrants (*PreC, Precore; C, Core; PreS, Presurface; S, Surface; X, X*) and flanking host genomes (position, chromosome, location of the genome, and gene names) are shown. The HBV genome became significantly methylated when integrated into highly methylated human genome regions, but not when integrated into unmethylated human genome regions. (×) The desired quantitative methylation levels were not obtained because of technical difficulties with the sequences that were being analyzed.

The dynamic changes in DNA methylation described here have a major functional impact on the biological behavior of HBV and underlie the molecular mechanisms that control infection or enable tumorigenesis. These findings may significantly impact public health given that millions of people worldwide are carriers of HBV. Distinct DNA methylation profiles may exist, for example, between primary HCCs in Japanese patients and those of other nationalities. Additional studies are needed to address this issue, and research into the influence of other environmental factors is required.

Increased viral DNA methylation is present in cancers associated with DNA viruses, including human papilloma virus types 16 and 18 (HPV 16 and 18) (Fernandez et al. 2009; Mirabello et al. 2012), Epstein-Barr virus (Uozaki and Fukayama 2008; Fernandez et al. 2009), and human T-lymphotropic virus 1 (Taniguchi et al. 2005). An analysis of the haplotype-resolved genome and epigenome of the aneuploid HeLa cervical cancer cell line revealed that an amplified, highly rearranged region of chromosome 8q24.21 harboring an integrated HPV18 genome likely represents the tumor-initiating event (Adey et al. 2013). Whether the dynamic changes in DNA methylation observed in cells with integrated HBV genomes also occur in human cells infected by other viruses is an interesting question for further study. We anticipate that our assay will be a powerful tool for this purpose and have successfully detected integrated HPV sequences in the genomes of cervical cancer cell lines (Y Watanabe, H Yamamoto, F Itoh, and N Suzuki, unpubl.).

This study provides novel mechanistic insights into HBV-mediated hepatocarcinogenesis, which may have preventive and therapeutic applications for carriers of HBV and patients with HBV-HCC, as it suggests that epigenetic alterations provide candidate biochemical markers and therapeutic targets. This study, together with a recent global survey of HBV integration events (Ding et al. 2012; Fujimoto et al. 2012; Jiang et al. 2012; Sung et al. 2012; Toh et al. 2013), provides a foundation for the further experimentation and mechanistic understanding of HBV-HCC.

Methods

Cell lines and primary tissues

The PLC/PRF/5 (Alexander) human hepatoma cell line was obtained from the Japanese Collection of Research Bioresources (JCRB). HepG2.2.15 cells, kindly gifted by Professor Stephan Urban

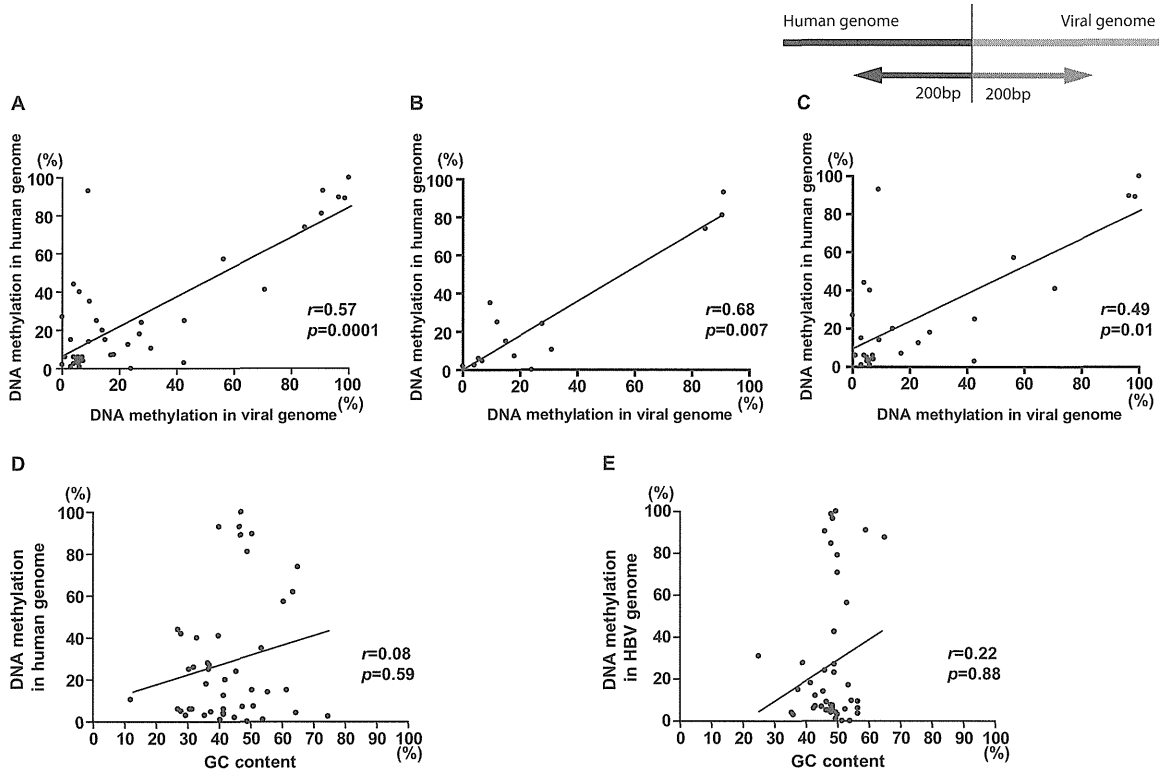

Figure 5. Correlation analysis between the methylation pattern of the integrated HBV DNA and that of the human genome. DNA fragments, including 200 bp of the HBV DNA and 200 bp of the human genome around the boundary, were analyzed for average methylation and GC content. (*A*) A correlation between the average methylation of the HBV DNA and that of the human genome in combined two cell lines and eight clinical samples ($n = 40$, $r = 0.57$, $P = 0.0001$, 95%CI = 0.3091–0.7545). (*B*) A correlation between the average methylation of the HBV DNA and that of the human genome in two cell lines ($n = 14$, $r = 068$, $P = 0.007$, 95%CI = 0.2233–0.8946). (*C*) A correlation between the average methylation of the HBV DNA and that of the human genome in eight clinical samples ($n = 26$, $r = 0.49$, $P = 0.01$, 95%CI = 0.1222–0.7463). (*D*) No correlation between the average methylation and GC contents in the human genome in the combined two cell lines and eight clinical samples ($n = 45$, $r = 0.08$, $P = 0.59$, 95%CI = −0.2253–0.3745). (*E*) No correlation between the average methylation and GC contents in the viral genome in the combined two cell lines and eight clinical samples ($n = 47$, $r = 0.22$, $P = 0.88$, 95%CI = −0.3151–0.2751).

at University Hospital Heidelberg, was derived from HepG2 cells transfected with a plasmid carrying four 5′-3′ tandem copies of the HBV genome (Koike et al. 1994). Cell lines were maintained in appropriate media containing 10% fetal bovine serum in plastic culture plates. Primary tissues from tumor and adjacent tissues were obtained at the time of the clinical procedures. Informed consent was obtained from all the patients before specimen collection. This study was approved by the institutional review board. DNA was extracted using the standard phenol–chloroform method. The concentration and quantity of extracted DNA were measured using a NanoDrop spectrophotometer (NanoDrop Technologies).

MCAM analysis

MCAM analysis was conducted as previously described (Oishi et al. 2012). A detailed protocol of MCA was previously described (Toyota et al. 1999). We used a custom human promoter array (G4426A-02212; Agilent Technologies) comprising 36,579 probes corresponding to 9021 unique genes. The probes on the array were selected to recognize SmaI/XmaI fragments mainly derived from sequences near gene transcription start sites. Five micrograms of genomic DNA was digested with 100 U of methylation-sensitive restriction endonuclease SmaI (New England Biolabs) for 24 h at 25°C, which cleaves unmethylated DNA leaving blunt ends (CCC/GGG). Subsequently, the DNA was digested with 20 U of methylation-insensitive restriction endonuclease XmaI for 6 h at 37°C, creating sticky ends (C/CCGGG). Five hundred milligrams of digested DNA was ligated using 50 μL of RMCA12 (5′-CCGGGCA GAAAG-3′)/RMCA24 (5′-CCACCGCCATCCGAGCCTTTCTGC-3′) primers and T4 DNA ligase (TaKaRa Bio) for 16 h at 16°C. After filling in the overhanging ends of the ligated DNA fragments at 72°C, the DNA was amplified for 5 min at 95°C followed by 25 cycles of 1-min incubation at 95°C and 3-min incubation at 77°C using 100 pmol of RMCA24 primer. MCA products were labeled with Cy5 (red) for DNA from hepatoma samples (both tumor and adjacent normal) and Cy3 (green) for DNA from human blood mixture of three healthy volunteers using a randomly primed Klenow polymerase reaction (Invitrogen) for 3 h at 37°C. Human CpG island arrays (4 × 44 K) were purchased from Agilent Technologies. Microarray protocols, including labeling, hybridization, and post-hybridization washing procedures, are provided at http://www.agilent.com/. Labeled samples were then hybridized to arrays in the presence of human Cot-1 DNA for 24 h at 65°C. After washing, arrays were scanned using an Agilent DNA microarray scanner and analyzed using Agilent Feature Extraction software (FE version 9.5.1.1, Agilent Technologies) at St. Marianna University School of Medicine. We used GeneSpring software (Agilent) for choosing candidate genes after normalization of the raw data.

DNA methylation analysis

Hidden Markov models have been successfully used to partition genomes into segments of comparable stochastic structure (Durbin et al. 1998). Using these models for sequence analysis performed