positive patients before treatment. For HBV resolved patients, monthly monitoring of HBV DNA levels is recommended during and for at least 1 year after the end of immunosuppressive therapy or chemotherapy. Preemptive antiviral therapy should be started as soon as possible if HBV DNA is detected during this monitoring; however, there is little evidence of HBV DNA monitoring to prevent hepatitis due to HBV reactivation in HBV resolved patients.

Reactivation of HCV infection

Although HCV reactivation is rare, hepatic toxicity related to chemotherapy is higher among patients with chronic HCV infection than in HCV uninfected patients,[26] suggesting that HCV reactivation occurred and can cause clinically relevant complications.

Hepatitis C virus-related liver dysfunction generally occurs 2–4 weeks after the cessation of chemotherapy.[27–30] A widely accepted hypothesis considering the pathogenesis indicates enhanced viral replication with a consequent increase in the number of infected hepatocytes following immunosuppressive treatment (Fig. 1). Withdrawal of immunosuppressive therapy leads to restoration of the host immune function, resulting in the rapid destruction of infected cells and hepatic injury.[27,31] Severe liver dysfunction was found to occur at a lower incidence in HCV positive patients than HBV positive patients.[5] The reason for this phenomenon is unknown; however, if severe hepatitis secondary to viral reactivation develops, mortality rates of HBV infected and HCV infected patients seem to be similar.[32–34]

CLINICAL EVIDENCE AND MANAGEMENT OF HCV REACTIVATION

Diagnosis for HCV reactivation

CHRONICALLY INFECTED PATIENTS have stable HCV RNA levels that may vary by approximately 0.5 $\log_{10}$ IU/mL;[35] therefore, an increase of the HCV viral load of more than 1 $\log_{10}$ IU/mL may be a sign of HCV reactivation. It was also reported that HCV reactivation showed an at least threefold increase in serum ALT in a patient in whom the tumor had not infiltrated the liver, who had not received hepatotoxic drugs and who had had no recent blood transfusions or other systemic infections besides HCV.[6,24] Changes in liver enzyme levels can be accompanied by the reappearance of HCV RNA or a sudden increase in the serum HCV RNA level.[6]

HCV reactivation after specific treatments

Patients with HCV infection who undergo HSCT or systematic chemotherapy including corticosteroids can experience severe hepatic dysfunction and fulminant hepatic failure (summarized in Table 2).

Corticosteroids have traditionally been associated with cases of HCV reactivation.[27,36] HCV reactivation has been associated with several immunosuppressive and chemotherapeutic agents, including rituximab, alemtuzumab, bleomycin, busulfan, cisplatin, cyclophosphamide, cyclosporin, cytarabine, dacarbazine, doxorubicin, etoposide, gemcitabine, methotrexate, vinblastine and vincristine;[27,37–44] however, many patients with HCV reactivation during treatment with one of these drugs were simultaneously treated with corticosteroids.[38,41,42,44,45] In a study by Zuckerman *et al.*,[46] 18 of 33 (54%) patients had mild to moderate increases of ALT, which occurred 2–3 weeks after the withdrawal of chemotherapy. HCV positive patients did not demonstrate a higher incidence of severe hepatic dysfunction during chemotherapy for malignancies than HCV negative patients; however, liver test abnormalities during therapy are very common and are seen in 54% HCV positive patients and in 36% HCV negative patients.

Whether corticosteroid therapy alone or in combination with other agents leads to reactivation of HCV infection and acute exacerbation of chronic HCV infection remains to be determined. A possible relationship between rituximab and HCV reactivation in patients with cancer has been reported.[41,44,45] Only the administration of rituximab-containing chemotherapy was associated with both acute exacerbation and reactivation of chronic HCV infection.[24]

Ennishi *et al.* also showed that the incidence of severe hepatic toxicity in HCV positive patients was significantly higher than in HCV negative patients, and HCV infection was determined to be a strong risk factor for this adverse effect in patients with diffuse large B-cell lymphoma (DLBCL) in the rituximab era.[44] These hepatic toxicities led to modification and discontinuation of immunochemotherapy, resulting in lymphoma progression. The study described that careful monitoring of hepatic function should be recommended for HCV positive patients, particularly those with high levels of pretreatment transaminase. More importantly, monitoring of HCV viral load demonstrated a marked enhancement of HCV replication, and it is suggested that increased HCV results in severe hepatic toxicity. Thus, HCV viral load should be

Table 2 Hepatic toxicity by HCV reactivation in HCV infected patients with hematological malignancies

Author	Year	Disease	Treatment	No. of cases with hepatic toxicity	Death from liver toxicity
Kanamori *et al.*	1992	AML	Allo-HSCT	2 patients	2
Maruta *et al.*	1994	AML, AA	Allo-HSCT	9 patients	2
Nakamura *et al.*	1996	Hematological malignancies	Various regimens	11 patients	5
Vento *et al.*	1996	B-cell NHL and HL	ABVD or CHOP- like regimen	2 patients	1
Luppi *et al.*	1998	B-cell NHL	Various regimens	20/35 patients	2
Zuckerman *et al.*	1998	Hematological malignancies	Various regimens	18/33 patients (55%)	0
Kawatani *et al.*	2001	Hematological malignancies	Various regimens	4/22 patients	1
Hamaguchi *et al.*	2002	Hematological malignancies	Allo-HSCT	40/58	9
Locasciulli *et al.*	2003	Hematological and solid malignancies	Allo-HSCT (21)/auto-HSCT (36)	21 6	2 1
Takai *et al.*	2005	Hematological malignancies	Various regimens	4/37 patients	0
Aksoy *et al.*	2006	DLBCL	Rituximab	0/1 patients	0
Besson *et al.*	2006	DLBCL	Various regimens	15/23 patients	3
Visco *et al.*	2006	DLBCL	CHOP-like, rituximab (35)	5/132 patients	1
Ennishi *et al.*	2008	DLBCL, MALT NHL	R-CHOP-like	1/5 patients	0
Hsieh *et al.*	2008	DLBCL	R-CNOP	1/1 patients	0
Ennishi *et al.*	2010	DLBCL	R-chemo	36/131 patients	6
Arcaini *et al.*	2010	NHL	R-chemo (28)	24/160 patients	3

AA, aplastic anemia; ABVD, doxorubicin hydrochloride (adriamycin), bleomycin, vinblastine and dacarbazine; Allo-HSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; auto-HSCT, autologous hematopoietic stem cell transplantation; CHOP, cyclophosphamide, vincristine and prednisolone; DLBCL, diffuse large B-cell lymphoma; MALT, extranodal-marginal zone lymphoma of the mucosa-associated lymphoid tissue; NHL, non-Hodgkin's lymphoma; R-chemo, rituximab plus steroids combined chemotherapy; R-CHOP, rituximab, cyclophosphamide, vincristine and prednisolone; R-CNOP, rituximab, cyclophosphamide, mitoxantrone, vincristine and prednisolone.

carefully monitored in HCV positive patients who receive immunochemotherapy.

Severity of HCV reactivation versus HBV reactivation

The health consensus regarding HCV reactivation seems to be less severe than that of HBV reactivation (summarized in Table 3). Previous reports described that the incidence of post-chemotherapy liver injury in HBV carriers was significantly higher than that in HCV carriers,[5,31,32] namely, the incidence of post-chemotherapy liver injury in 25 HBV carriers (36%) was significantly higher than that in 37 HCV carriers (10.8%, $P = 0.026$),[31] and 44 (51.8%) of the 85 patients reported to have severe hepatitis along with hematological malignancies were HBV carriers, while only 11 (12.9%) were HCV carriers;[32] however, the mortality rates did not differ between HBV and HCV carriers (40.9% vs 45.5%) once severe hepatitis developed.

In a large Italian study of 57 HCV infected patients who underwent HSCT, patients undergoing autologous HSCT had a significantly lower risk of reactivation post-transplant than the allogeneic group (16% vs 100%, $P = 0.004$). In the allogeneic HSCT group, HCV reactivation occurred mainly within 6 months after HSCT, whereas in the autologous group, reactivation occurred within the first 3 months post-transplant. In this cohort, one HBsAg positive and three anti-HCV positive patients before HSCT died of liver failure. The risk of death from liver failure was not significantly different between HBsAg and anti-HCV positive patients, being 3% and 8% at 24 months, respectively ($P = 0.6$), or between recipients of autologous (5%) and allogeneic HSCT (7%) ($P = 0.34$).[33]

In a Japanese multicenter study of 135 patients with HBV or HCV infection who received allogeneic transplants, transient hepatitis was more common in HBV infected patients than in HCV infected patients, but the rates of fulminant hepatitis and death due to hepatic failure were similar in both groups.[34]

Table 3 Clinical state by HCV reactivation versus HBV reactivation

Severity or prognosis	Year	Survey period	No. of patients	Country	% of HBV	% of HCV	*P*	Comments	Reference
Liver injury	2005	1996–2002	601	Japan	36% (9/25)	10.8% (4/37)	0.026	Patients with hematological malignancies	31
Severe hepatitis	1996	1987–1991	Surveillance in 250 hospitals	Japan	51.8% (44/85)	12.9% (11/85)	ND	In 85 patients having severe hepatitis along with hematological malignancies	32
Death from liver failure	2003	1996–2000	90	Italy	3% (1/33)	8% (3/38*)	0.6	Patients with HBV or HCV receiving HSCT (during 24 months)	33
Hepatic failure	2002	1986–1998	135	Japan	10% (8/77)	12% (7/58)	ND	Patients with HBV or HCV receiving HSCT	34

HBV, hepatitis B virus; HCV, hepatitis C virus; HSCT, hematopoietic stem cell transplant; ND, not done.
*Fifty-seven were anti-HCV positive; of these, 38 were also tested for HCV RNA.

Outcome of HCV infected hematological patients

As previously highlighted, there is no significant short-term impact of HCV on the outcome after HSCT. Nevertheless, the long-term impact of chronic HCV infection can be deleterious in the liver, causing significant fibrosis progression, liver failure and increased risk of hepatocellular carcinoma (HCC). One study reported the rapid progression of hepatitis C in patients with humoral immunodeficiency disorders.[47] Another group has recently reported a more rapid rate of fibrosis progression after HSCT, with median time to cirrhosis of 18 years, as compared to 40 years seen in the control group. HCV disease progression ranked third, behind infections and GVHD, as a cause of late death after HSCT.[48] Long-term survivors after HSCT thus appear to be at higher risk for HCV-related complications and treatment of HCV becomes critical. A possible explanation for the genesis of cirrhosis could be an immune imbalance or impaired regulation of B and T cells.[47,48]

In various regimens for hematological malignancies, Ennishi *et al.* reported that hepatic disease progressed in four patients, and HCC was found to increase the risk of death from hepatic failure significantly in lymphoma patients receiving conventional chemotherapy, even during short-term observation.[44] Cox multivariate analysis showed that older age and advanced stage had significant adverse effects on overall survival (OS); however, HCV infection was not associated with poor progression-free survival (PFS) or OS. Besson *et al.* described that the overall proportion of subjects undergoing hepatic toxicity was 65% (15/23 patients). Outcome of HCV positive patients was poorer for OS ($P = 0.02$), but not for event-free survival ($P = 0.13$).[49] Visco *et al.* also described that only five of 132 patients (4%) had to discontinue chemotherapy due to severe liver function impairment.[50] Although previous papers mentioned that rituximab induced HCV reactivation after spontaneous remission in DLBCL,[45,51] the addition of rituximab did not seem to affect patients' tolerance to treatment. Five-year overall survival of the entire cohort was 72%, while 5-year PFS of the 132 patients treated with intent to cure was 51%. The prognosis of HCV infected patients with DLBCL is still controversial.

Recently, Arcaini *et al.*[43] studied 160 HCV positive patients with NHL (59 indolent NHL, 101 aggressive). Among 28 patients treated with rituximab-containing chemotherapy, five (18%) developed liver toxicity, and among 132 independent patients who received chemotherapy, only nine (7%) had hepatotoxicity, suggesting

that rituximab was related to a slightly higher occurrence of toxicity. Median PFS for patients who experienced liver toxicity was significantly shorter than median PFS of patients without toxicity (2 and 3.7 years, respectively, $P = 0.03$). HCV infected patients with NHL developed liver toxicity significantly, often leading to interruption of treatment.

Based on these findings, the impact of HCV infection on the outcome after HSCT or rituximab-containing chemotherapy seems to be deleterious for OS but not for event-free survival. Further studies are required in prospective multicenter cohorts.

Treatment of HCV infected patients with hematological malignancies

The long-term impact of chronic HCV infection can be deleterious to the liver, causing significant fibrosis progression, liver failure and increased risk of HCC. Interestingly, a more rapid rate of fibrosis progression was reported after HSCT.[48] Therapy for HCV infection in patients with hematological malignancy can be considered once a patient's immunity and bone marrow have recovered, immunosuppressive drugs have been stopped, and there is no evidence of GVHD, because the hematological adverse effects of anti-HCV drugs can exacerbate the toxicity of chemotherapy, which can involve complications such as severe cytopenias and potentially life-threatening infections.[52] Overall, antiviral therapy for HCV in patients (e.g. HIV, transplant) is often associated with poor response rates, even though patients with chronic HCV infection were treated with the combination of pegylated interferon-α and ribavirin.[53–55] The use of direct-acting antiviral drugs (such as recently approved inhibitors of nonstructural protein 3/4A [NS3/4A] protease [boceprevir or telaprevir], or NS5B polymerase inhibitors) has not been evaluated in patients with cancer. Boceprevir and telaprevir can inhibit hepatic drug-metabolizing enzymes such as cytochrome P450 (CYP)2C, CYP3A4 or CYP1A;[56] therefore, these agents potentially interact with various drugs that are co-administrated in patients with cancer. These new antiviral drugs should be used with caution in patients with cancer.

Large-scale studies are needed to better define which patients with cancer are most likely to benefit from simultaneous antiviral therapy and cytotoxic chemotherapy. Notably, antiviral treatment with pegylated interferon-α and ribavirin should not be used early in the post-transplant period (<2 years after transplantation) in patients who have undergone allogeneic HSCT as interferon-α therapy may precipitate or induce the development of GVDH.[57]

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2′-Fluoro-6′-methylene-carbocyclic adenosine phosphoramidate (FMCAP) prodrug: In vitro anti-HBV activity against the lamivudine–entecavir resistant triple mutant and its mechanism of action

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ABSTRACT

Novel 2′-fluoro-6′-methylene-carbocyclic adenosine (FMCA) monophosphate prodrug (FMCAP) was synthesized and evaluated for its in vitro anti-HBV potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). FMCA demonstrated significant antiviral activity against wild-type as well as lamivudine–entecavir resistant triple mutant (L180M + M204V + S202G). The monophosphate prodrug (FMCAP) demonstrated greater than 12-fold (12×) increase in anti-HBV activity without increased cellular toxicity. Mitochondrial and cellular toxicity studies of FMCA indicated that there is no significant toxicity up to 100 μM. Mode of action studies by molecular modeling indicate that the 2′-fluoro moiety by hydrogen bond as well as the Van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug-resistant mutants.

The chronic HBV infection is strongly associated with liver diseases like chronic hepatic insufficiency, cirrhosis and hepatocellular carcinoma (HCC).[1] According to the World Health Organization (WHO), currently about 2 billion people world-wide have been infected with HBV and more than 350 million live with chronic infection. Acute or chronic outcomes of HBV infection are estimated to cause the deaths of 600,000 people worldwide every year.[2]

Currently, there are several nucleos(t)ide analogues available to treat chronic hepatitis B virus infection.[3–6] The major target of these drugs is to inhibit the viral reverse transcriptase (RT)/DNA polymerase, which is responsible for the synthesis of the minus-strand DNA. Although the currently used agents are well tolerated and effective in suppressing the viral replication for extended periods, the significant rate of virological relapse caused by drug resistance remains a critical issue.

Lamivudine (LVD) was first introduced as the orally active anti-HBV agent in 1998. Lamivudine profoundly suppresses HBV replication in patients with chronic hepatitis B infection; however, lamivudine-resistant HBV (LVDr) was isolated from a significant numbers of patients during the treatment with lamivudine. Currently, there are several antiviral options exist for these patients viz., to use adefovir or high dose (1.0 mg/day) of entecavir, or more recently tenofovir. However, this resulted in also the development of resistance mutants during the long term therapy. At present, entecavir is the most prescribed drug, and is recommended for patients with the wild-type as well as for those harboring adefovir and lamivudine-resistant strains. However, recent clinical studies by Tanaka and his co-workers suggested that the entecavir mutant in the lamivudine-resistant patients (L180M + M204V + S202G) causes a viral breakthrough: 4.9% of patients at baseline increases to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.[7] Therefore, it is of great interest to discover novel anti-HBV agent, which is effective against lamivudine- and entecavir-resistant triple mutants (L180M + M204V + S202G).

The potency of a nucleos(t)ide analogue is determined by its ability to serve as a competitive inhibitor of the HBV polymerase relative to that of the natural substrate, the nucleotide triphosphate.[8] However, host cellular kinases limit the pharmacological potency of nucleoside analogues by phosphorylation to their corresponding triphosphates. Particularly, the initial kinase action on the nucleoside to the monophosphate is the rate-limiting step. However, many synthetic nucleosides are not phosphorylated or the rate of phosphorylation is very slow due to the structural requirement of the kinases, resulting in only generating a low quantity of the triphosphate. To overcome this phosphorylation issue, nucleoside phosphoramidate prodrugs have been introduced,[8,9] which

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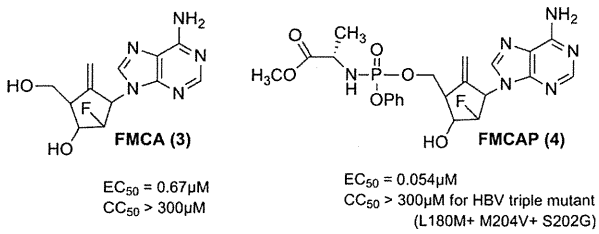


Figure 1. Structures of 2′-fluoro-6′-methylene–carbocyclic adenosine (FMCA; **3**) and its prodrug (FMCAP; **4**).

can bypass the rate-limiting first step of monophosphorylation. Phosphoramidate prodrugs have demonstrated to enhance the nucleoside potency in cell culture as well as in patients.[10,11] This methodology greatly increases the lipophilicity of the nucleotide to increase the cell penetration as well as to target the liver cells in vivo.

In this communication, we present that a FMCA phosphoramidate prodrug is such an agent, which can potentially be used for the treatment of patients who experience viral breakthrough due to the triple mutants caused by the use of lamivudine and entecavir.

In our previous report, we have demonstrated that the novel carbocyclic adenosine analog **3** (FMCA Fig. 1) exhibits significant anti-HBV activity against wild type as well as adefovir/lamivudine resistant strains.[12] The present study describes the synthesis and antiviral evaluation of a phosphoramidate of FMCA (FMCAP), which demonstrated the significantly improved in vitro potency. Additionally, we studied its mechanism of action how FMCA-TP can effectively bind to the HBV polymerase by molecular modeling and still exerts the antiviral activity against the lamivudine–entecavir triple mutant (L180M + M204V + S202G).

FMCAP (**4**, Scheme 1)[13] was synthesized using a known method in the literature,[14,15] in which the phosphorylation of phenol with phosphorus oxychloride generates phenyl dichlorophosphate **1**, which was coupled with L-alanine methyl ester in the presence of *tri*-ethyl amine in dichloromethane to give chlorophosphoramidate reagent **2**, which, in turn, was coupled with FMCA **3** in the presence of 1-methyl imidazole in THF to furnish the phosphoramidate **4** in good yield.

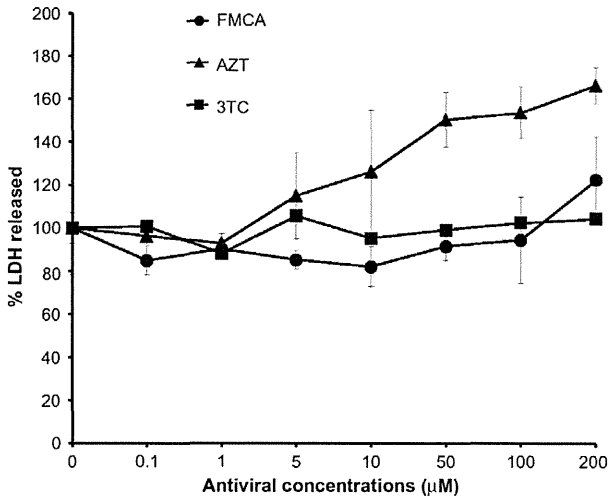
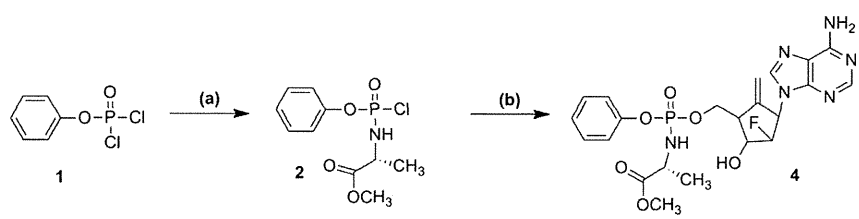


Figure 2. Mitochondrial toxicity of FMCA **3**, AZT and 3TC through lactate dehydrogenase release (LDH) assay.

FMCA **3** and FMCAP **4** were evaluated in vitro against the wild-type as well as the lamivudine–entecavir resistant clone (L180M + S202I + M202V). The FMCA **3** and FMCAP **4** demonstrated significant anti-HBV activity (EC_{50} 0.548 ± 0.056 & 0.062 ± 0.011 μM, respectively) against the wild-type virus, while lamivudine and entecavir also demonstrated potent anti-HBV activity (EC_{50} 0.056 ± 0.003 & 0.008 μM, respectively) (Table 1). It is noteworthy to mention that the anti-HBV potency of FMCAP (**4**) was increased to eight-fold (8×) in comparison to that of FMCA **3**, which indicates the importance of the initial phosphorylation of the nucleoside.

FMCA **3** and FMCAP **4** were further evaluated for their in-vitro antiviral potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). It was observed that the anti-HBV potency of both FMCA **3** and FMCAP **4** (EC_{50} 0.67 & 0.054 μM, respectively) were maintained against the resistant clone, and furthermore, the anti-HBV activity of FMCAP **4** was enhanced a 12-fold (12×) with respect to that of FMCA without significant enhancement of cellular toxicity. It was also noteworthy to mention that the anti-HBV potency of entecavir against the mutant



Scheme 1. Reagent and conditions: (a) L-alanine methyl ester hydrochloride, Et_3N, CH_2Cl_2; (b) FMCA (3), NMI, THF, rt overnight.

Table 1
In vitro anti-HBV activity of FMCA **3**, FMCAP **4**, lamivudine and entecavir against wild-type and entecavir drug-resistant mutant (L180M + M204V + S202G) in Huh7 cells

Compounds	HBV Strains			
	Wild-type			L180M + M204V + S202G
	EC_{50} (μM)	EC_{90} (μM)	CC_{50} (μM)	EC_{50} (μM)
FMCA **3**	0.548 ± 0.056	6.0 ± 0.400	>300	0.67
FMCAP **4**	0.062 ± 0.011	0.46 ± 0.060	>300	0.054
Lamivudine	0.056 ± 0.003	0.142 ± 0.008	>300	>500[17]
Entecavir	0.008	0.033	28	1.20[16]

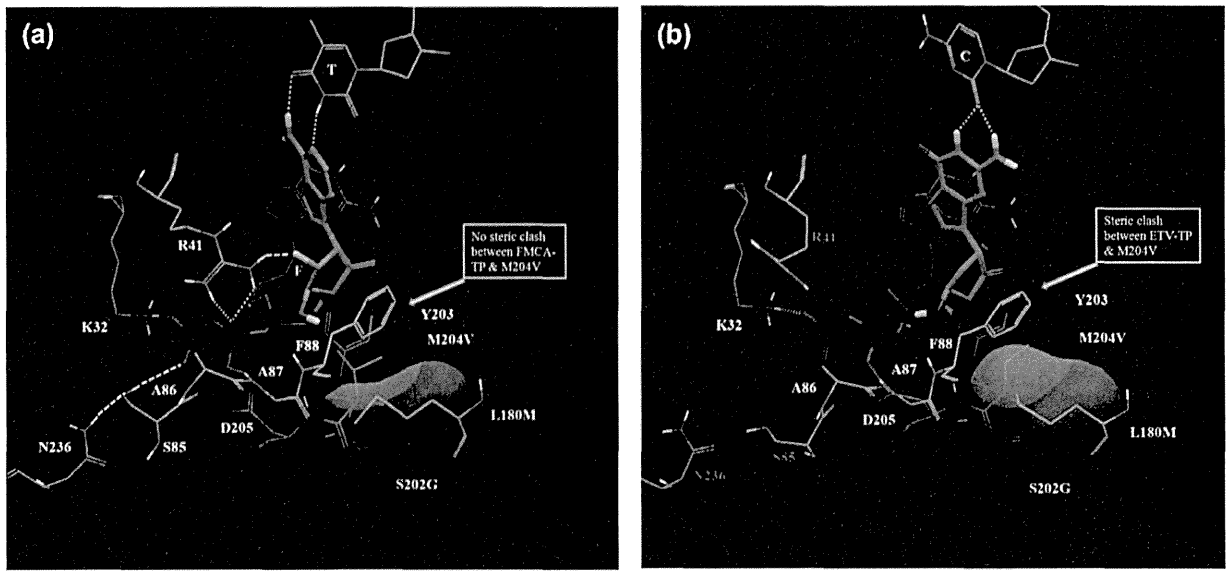


Figure 3. (a) FMCA-TP binding mode in ETVr (L180M + M204V + S202G); and (b) ETV-TP binding mode in ETVr (L180M + M204V + S202G) and there is a steric hindrance. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å). The Van der Waals surface of L180M is colored yellow. The Van der Waals surface of M204V is shown in spring green. The Van der Waals surface of S202G is colored orange. The exocyclic double bond is shown blue color.

Table 2
MBAE (multi-ligand bimolecular association with energetics) calculation of FMCA-TP and ETV-TP after Glide XP docking[21] and energy minimization[22]

Strains	Compounds	Energy difference results (ΔE, kcal/mol)		
		Total energy	VdW[a]	Electrostatic
Wild-type	FMCA-TP	−588.05	375.78	−6341.08
	ETV-TP	−597.25	350.35	−6009.65
ETVr	FMCA-TP	−591.54	359.91	−6245.68
(L180M + M204V + S202G)	ETV-TP	−320.28	248.82	−4831.12

[a] Van der Waals interaction.

was reduced by 150-fold (EC_{50} 1.2 μM) in comparison to wild type.[16]

In the preliminary mitochondrial toxicity studies in HepG2 cells by measuring the lactic dehydrogenase release,[18] FMCA **3** did not exhibit any significant toxicity up to 100 μM like lamivudine (3TC), while azidothymidine (AZT) shows the increase of toxicity (Fig. 2).

In our previous report, we described molecular modeling studies for favorable anti-HBV activity of FMCA-TP in wild-type as well as in N236T adefovir resistant (ADVr) mutant.[12] In the current studies, it was of interest to know how the FMCA and its prodrug maintain the anti-HBV activity against ETVr triple mutant (L180M + M204V + S202G) in comparison to entecavir. Therefore, molecular modeling studies were conducted to obtain the insight of the molecular mechanism of FMCA-TP by using the Schrodinger Suite modules.[19] A previously described homology model was used to further explore the impact of the ETVr to the HBV-RT.[12] The homology model of HBV-RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD).[20]

The binding mode of FMCA-TP and ETV-TP in ETVr (L180M + M204V + S202G) HBV-RT are depicted in Figure 3a and b, respectively. Their MBAE (multi-ligand biomolecular association with energetics)[22] calculations of FMCA-TP (total energy, wt −588.05 & ETVr −591.54 kcal/mol) and ETV-TP (total energy, wt −597.25 & ETVr −320.28 kcal/mol) after glide XP (extra precision) docking[21] and energy minimization in ETVr HBV-RT are shown in Table 2. The triphosphate of FMCA-TP forms all the network of hydrogen bonds with the active site residues (Fig. 3a), K32, R41, S85 & A87 in the similar manner as in wild-type,[12] whereas ETV-TP lose the hydrogen bonding with R41 & S85. The γ-phosphate of FMCA-TP maintains a critical H-bonding with the OH of S85 with connection of hydrogen bonds between S85 and N236 in ETVr HBV-RT also. However, γ-phosphate ETV-TP does not maintain this critical H-bonding with S85 and N236 (Fig. 3b).

The carbocyclic ring with an exocyclic double bond of FMCA-TP and ETV-TP makes the favorable Van der Waals interaction with F88 in ETVr HBV-RT (Fig. 3a and b). There is no steric clash in between exocyclic double bond of FMCA-TP and M204V residue, whereas ETV-TP exocyclic double bond has steric clash with M204V residue in ETVr HBV-RT. The 2′-fluorine substituent in the carbocyclic ring of FMCA-TP appears to promote an additional binding with the NH of R41 guanidino group as shown in Figure 3a, which is in agreement with the antiviral activity of FMCA-TP shown in Table 1. Overall, the modeling studies can qualitatively explain the favorable anti-HBV activity of FMCA-TP against ETVr mutant (L180M + M204V + S202G) in comparison to entecavir as shown in Table 1.

In conclusion, 2′-fluoro-6′-methylene-carbocyclic adenosine phosphoramidate prodrug (FMCAP) was synthesized, which demonstrated the significantly increased anti-HBV potency relative to the parent compound, FMCA in vitro. Molecular modeling studies delineated the mechanism of FMCA-TP and how it can effectively bind to the lamivudine–entecavir resistant triple mutant resulting

in maintaining the anti-HBV activity against the mutant. Furthermore, FMCA has been studied for the release of lactic dehydrogenase for potential mitochondrial toxicity and found that no significant increase of toxicity of FMCA compared with other commonly used anti-HIV nucleoside drugs. Very recently, a preliminary in vivo study in chimeric mice harboring the triple mutant, FMCAP was found to reduce HBV viral load while entecavir did not (data not shown). In view of these promising anti-HBV activities and non-toxicity of FMCAP as well as the interesting mechanism of antiviral activity, the chiral synthesis of FMCAP and its mitochondrial toxicity studies for preclinical investigation are warranted.

Acknowledgment

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13. *Compound* ***4***: ^{1}H NMR (500 Mz, $CDCl_3$) δ 8.35 (s, 1H), 7.86 (d, J = 3.0 Hz, 1H), 7.34–7.15 (m, 5H), 5.95 (m, 3H), 5.26 (d, J = 8.0 Hz, 1H), 5.01–4.90 (m, 1H), 4.83 (s, 1H), 4.50–4.41 (m, 2H), 4.25–4.04 (m, 3H), 3.71 (s, 3H), 3.07 (s, 1H), 1.40 (d, J = 6.5 Hz, 3 H); ^{19}F NMR (500 MHz, $CDCl_3$) δ −192.86 (m, 1F); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171, 159.0, 156.5, 152.5, 150.4, 142.9, 130.1, 121.2, 120.3, 106.7, 102.4, 72.2, 71.1, 62.3, 51.9, 46.3, 43.9, 19.1; ^{31}P NMR (202 MHz, $CDCl_3$): δ 2.67, 2.99. Anal. Calcd For $C_{22}H_{26}FN_6O_6P.0.5H_2O$: C, 49.91; H, 5.14; N, 15.87; Found C, 49.84; H, 5.06; N, 15.22.
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Soluble MICA and a *MICA* Variation as Possible Prognostic Biomarkers for HBV-Induced Hepatocellular Carcinoma

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Abstract

MHC class I polypeptide-related chain A (MICA) molecule is induced in response to viral infection and various types of stress. We recently reported that a single nucleotide polymorphism (SNP) rs2596542 located in the *MICA* promoter region was significantly associated with the risk for hepatitis C virus (HCV)-induced hepatocellular carcinoma (HCC) and also with serum levels of soluble MICA (sMICA). In this study, we focused on the possible involvement of MICA in liver carcinogenesis related to hepatitis B virus (HBV) infection and examined correlation between the *MICA* polymorphism and the serum sMICA levels in HBV-induced HCC patients. The genetic association analysis revealed a nominal association with an SNP rs2596542; a G allele was considered to increase the risk of HBV-induced HCC ($P = 0.029$ with odds ratio of 1.19). We also found a significant elevation of sMICA in HBV-induced HCC cases. Moreover, a G allele of SNP rs2596542 was significantly associated with increased sMICA levels ($P = 0.009$). Interestingly, HCC patients with the high serum level of sMICA (>5 pg/ml) exhibited poorer prognosis than those with the low serum level of sMICA (≤5 pg/ml) ($P = 0.008$). Thus, our results highlight the importance of *MICA* genetic variations and the significance of sMICA as a predictive biomarker for HBV-induced HCC.

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Introduction

Hepatocellular carcinoma (HCC) reveals a very high mortality rate that is ranked the third among all cancers in the world [1]. HCC is known to develop in a multistep process which has been related to various risk factors such as genetic factors, environment toxins, alcohol and drug abuse, autoimmune disorders, elevated hepatic iron levels, obesity, and hepatotropic viral infections [2]. Among them, chronic infection with hepatitis B virus (HBV) is one of the major etiological factors for developing HCC with considerable regional variations ranging from 20% of HCC cases in Japan to 65% in China [3].

Interestingly, clinical outcome after the exposure to HBV considerably varies between individuals. The great majority of individuals infected with HBV spontaneously eliminate the viruses, but a subset of patients show the persistent chronic hepatitis B infection (CHB), and then progresses to liver cirrhosis and HCC through a complex interplay between multiple genetic and environmental factors [4]. In this regard, genome wide association studies (GWAS) using single nucleotide polymorphisms (SNPs) have highlighted the importance of genetic factors in the pathogenesis of various diseases including CHB as well as HBV-induced HCC [5,6,7,8,9,10,11,12,13]. Recently, we identified a genetic variant located at 4.7 kb upstream of the *MHC class I polypeptide-related chain A* (*MICA*) gene to be strongly associated with hepatitis C virus (HCV) -induced HCC development [14].

MICA is highly expressed on viral-infected cells or cancer cells, and acts as ligand for NKG2D to activate antitumor effects of Natural killer (NK) cells and $CD8^+$ T cells [15,16]. Our previous results indicated that a G allele of SNP rs2596542 was significantly associated with the lower cancer risk and the higher level of soluble MICA (sMICA) in the serum of HCV-induced HCC patients, demonstrating the possible role of MICA as a tumor suppressor. However, elevation of serum sMICA was shown to be associated with poor prognosis in various cancer patients [17,18,19,20].

Matrix metalloproteinases (MMPs) can cleave MICA at a transmembrane domain [21] and release sMICA proteins from cells. Since sMICA was shown to inhibit the antitumor effects of NK cells and $CD8^+$ T cells by reduction of their affinity to binding to target cells [22,23], the effect of MICA in cancer cells would be modulated by the expression of MMPs. To elucidate the role of MICA in HBV-induced hepatocellular carcinogenesis, we here report analysis of the *MICA* polymorphism and serum sMICA level in HBV-induced HCC cases.

Materials and Methods

Study participants

The demographic details of study participants are summarized in Table 1. A total of 181 HCC cases, 597 CHB patients, and 4,549 non-HBV controls were obtained from BioBank Japan that was initiated in 2003 with the funding from the Ministry of Education, Culture, Sports, Science and Technology, Japan [24]. In the Biobank Japan Project, DNA and serum of patients with 47 diseases were collected through collaborating network of 66 hospitals throughout Japan. List of participating hospitals is shown in the following website (http://biobankjp.org/plan/member_hospital.html). A total of 226 HCC cases, 102 CHB patients, and 174 healthy controls were additionally obtained from the University of Tokyo. The diagnosis of chronic hepatitis B was conducted on the basis of HBsAg-seropositivity and elevated serum aminotransferase levels for more than six months according to the guideline for diagnosis and treatment of chronic hepatitis (The Japan Society of Hepatology, http://www.jsh.or.jp/medical/gudelines/index.html). Control Japanese DNA samples (n = 934) were obtained from Osaka-Midosuji Rotary Club, Osaka, Japan. All HCC patients were histopathologically diagnosed. Overall survival was defined as the time from blood sampling for sMICA test to the date of death due to HCC. Patients who were alive on the date of last follow-up were censored on that date. All participants provided written informed consent. This research project was approved by the ethics committee of the University of Tokyo and the ethics committee of RIKEN. All clinical assessments and specimen collections were conducted according to Declaration of Helsinki principles.

SNP genotyping

Genotyping platforms used in this study were shown in Table 1. We genotyped 181 HCC cases and 5,483 non-HBV control samples using either Illumina Human Hap610-Quad or Human Hap550v3. The other samples were genotyped at SNP rs2596542 by the Invader assay system (Third Wave Technologies, Madison, WI).

MICA variable number tandem repeat (VNTR) locus genotyping

Genotyping of the *MICA* VNTR locus in 176 HBV-induced HCC samples was performed using the primers reported previously by the method recommended by Applied Biosystems (Foster City, CA) [14]. Briefly, the 5′ end of forward primer was labeled with 6-FAM, and reverse primer was modified with GTGTCTT non-random sequence at the 5′ end to promote Plus A addition. The PCR products were mixed with Hi-Di Formamide and GeneScan-600 LIZ size standard, and separated by GeneScan system on a 3730×l DNA analyzer (Applied Biosystems, Foster City, CA). GeneMapper software (Applied Biosystems, Foster City, CA) was employed to assign the repeat fragment size (Figure S1).

Quantification of soluble MICA

We obtained serum samples of 111 HBV-positive HCC samples, 129 HCV-positive HCC samples, and 60 non-HBV controls from Biobank Japan. Soluble MICA levels were measured by sandwich enzyme-linked immunosorbent assay, as described in the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

The association between an SNP rs2596542 and HBV-induced HCC was tested by Cochran-Armitage trend test. The Odds ratios were calculated by considering a major allele as a reference. Statistical comparisons between genotypes and sMICA levels were performed by Kruskal-Wallis test (if more than two classes for comparison) or Wilcoxon rank test using R. Overall survival rate of the patients was analyzed by Kaplan-Meier method in combination with log-rank test with SPSS 20 software. The period for the survival analysis was calculated from the date of blood sampling to the recorded date of death or the last follow-up date. Differences with a P value of <0.05 were considered statistically significant.

Results

Association of SNP rs2596542 with HBV-induced HCC

In order to examine the effect of rs2596542 genotypes on the susceptibility to HBV-induced HCC, a total of 407 HCC cases and 5,657 healthy controls were genotyped. The Cochran Armitage trend test of the data revealed a nominal association

Table 1. Demographic details of subjects analyzed.

Subjects	Source	Genotyping platform	Number of Sample	Female (%)	Age (mean+/−sd)
Liver Cancer	BioBank Japan	Illumina Human Hap610-Quad	181	17.9	62.94±9.42
	University of Tokyo	Invader assay	226		
Control	BioBank Japan	Illumina Human Hap550v3	4549	47.95	55.19±12.5
	Osaka**	Illumina Human Hap550v3	934		
	University of Tokyo	Invader assay	174		
Chronic hepatitis B*	BioBank Japan	Invader assay	597	45.66	61.31±12.6
	University of Tokyo	Invader assay	102		

*Chronic hepatitis B patients without liver cirrhosis and liver cancer during enrollment.
**Healthy volunteers from Osaka Midosuji Rotary Club, Osaka, Japan.
doi:10.1371/journal.pone.0044743.t001

between HBV-induced HCC and rs2596542 in which a risk allele G was more frequent among HBV-induced HCC cases than an A allele (P = 0.029, OR = 1.19, 95% CI: 1.02–1.4; Table 2). To further investigate the effect of rs2596542 on the progression from CHB to HBV-induced HCC, we genotyped a total of 699 CHB cases without HCC. Although the progression risk from CHB to HBV-induced HCC was not statistically significant with rs2596542 (P = 0.197 by the Cochran Armitage trend test with an allelic OR = 1.3 (0.94–1.36); Table 2), we found a similar trend of association in which the frequency of a risk-allele G was higher among HBV-induced HCC patients than that of CHB subjects. Since we previously revealed that an A allele was associated with a higher risk of HCV-induced HCC with OR of 1.36 [14], the s2596542 alleles that increased the risk of HCC were opposite in HBV-induced HCC and HCV-induced HCC.

Soluble MICA levels are associated with SNP rs2596542

We subsequently performed measurement of soluble MICA (sMICA) in serum samples using the ELISA method in 176 HBV-positive HCC cases and 60 non-HBV controls. Nearly 30% of the HBV-induced HCC cases revealed the serum sMICA level of >5 pg/ml (defined as high) while the all control individuals except one showed that of ≤5 pg/ml (defined as low) ($P = 4.5 \times 10^{-6}$; Figure 1A). Then, we examined correlation between SNP rs2596542 genotypes and serum sMICA levels in HBV-positive HCC cases. Interestingly, rs2596542 genotypes were significantly associated with serum sMICA levels (P = 0.009; Figure 1B); 39% of individuals with the GG genotype and 20% of those with the AG genotype were classified as high for serum sMICA, but only 11% of those with the AA genotype were classified as high (AA+AG vs GG; P = 0.003) (Figure 1B). These findings were similar with our previous reports in which a G allele was associated with higher serum sMICA levels in HCV-induced HCC patients [14].

Negative association of variable number of tandem repeat (VNTR) with sMICA level

The *MICA* gene harbors a VNTR locus in exon 5 that consists of 4, 5, 6, or 9 repeats of GCT as well as a G nucleotide insertion into a five-repeat allele (referred as A4, A5, A6, A9, and A5.1, respectively). The insertion of G (A5.1) causes a premature translation termination and results in loss of a transmembrane domain, which may produce the shorter form of the MICA protein that is likely be secreted into serum [25]. However, the association of this VNTR locus with serum sMICA level was controversial among studies [14,26,27,28]. Therefore, we examined the association between the VNTR locus and sMICA level in HBV-induced HCC patients, and found no significant association (Figure S1 and S2), concordant with our previous report for HCV-induced HCC patients [14].

Soluble MICA levels are associated with survival of HCC patients

In order to evaluate the prognostic significance of serum sMICA levels in HCC patients, we performed survival analysis of HCC patients. A total of 111 HBV-infected HCC patients and 129 HCV-infected HCC patients were included in this analysis. The mean survival period for HBV- and HCV-infected patients with less than 5 pg/ml of serum sMICA were 67.1 months (95% CI: 61.1–73.1, n = 83), and 58.2 months (95% CI: 51.4–65.0, n = 85), respectively. On the other hand, for patients with more than 5 pg/ml of serum sMICA, the mean survival periods were 47.8 months (95% CI: 34.8–30.9, n = 28) for HBV-induced HCC patients and 59.5 months (95% CI: 51.9–67.1, n = 44) for HCV-induced HCC patients. The Kaplan-Maier analysis and log-rank test indicated that among HBV-induced HCC subjects, the patients in the high serum sMICA group showed a significantly shorter survival than those in the low serum sMICA (P = 0.008; Figure 2). In addition, we performed multi-variate analysis to test whether sMICA is an independent prognostic factor by including age and gender as covariates. The results revealed significant association of sMICA levels with overall survival (P = 0.017) but not with age and gender (Table S1).However, we found no association between the serum sMICA level and the overall survival in the HCV-induced HCC subjects (P = 0.414; Figure S3). Taken together, our findings imply the distinct roles of the *MICA* variation and sMICA between HBV- and HCV-induced hepatocellular carcinogenesis.

Vascular invasion in HBV-related HCC patients is associated with soluble MICA levels

Since sMICA levels were associated with the overall survival of HBV-related HCC patients, we tested whether sMICA levels affect survival through modulating invasive properties of tumors or size of the tumors. We tested the association between sMICA levels and vascular invasion in 35 HBV-related HCC cases, among whom 7 cases were positive and 21 cases were negative for vascular invasion. We found significant association between sMICA levels and vascular invasion (Figure 3; P = 0.014) in which 7 cases with positive vascular invasion showed high levels of sMICA (mean = 54 pg/ml) than 21 cases without vascular invasion (mean = 7.51 pg/ml). However, we found no association between tumor size and sMICA levels (P = 0.56; data not shown). These results suggest that sMICA may reduce the survival of HBV-related HCC patients by affecting the invasive properties of tumors.

Discussion

Several mechanisms such as HBV-genome integration into host chromosomal DNA [29] and effects of viral proteins including HBx [30] are shown to contribute to development and progression of HCC, while the immune cells such as NK and T cells function as key antiviral and antitumor effectors. MICA protein has been

Table 2. Association between HCC and rs2596542.

SNP	Comparison	Chr	Locus	Case MAF	Control MAF	P*	OR*	95% CI
rs2596542	HCC vs. Healthy control	6	*MICA*	0.294	0.332	0.029	1.19	1.02–1.4
rs2596542	HCC vs. CHB	6	*MICA*	0.294	0.320	0.197	1.13	0.94–1.36

Note: 407 HCC cases, 699 CHB subjects and 5,657 non-HBV controls were used in the analysis.
Chr.,chromosome; MAF, minor allele frequency; OR, odds ratio for minor allele; CI, confidence interval.
*Obtained by Armitage trend test.
doi:10.1371/journal.pone.0044743.t002

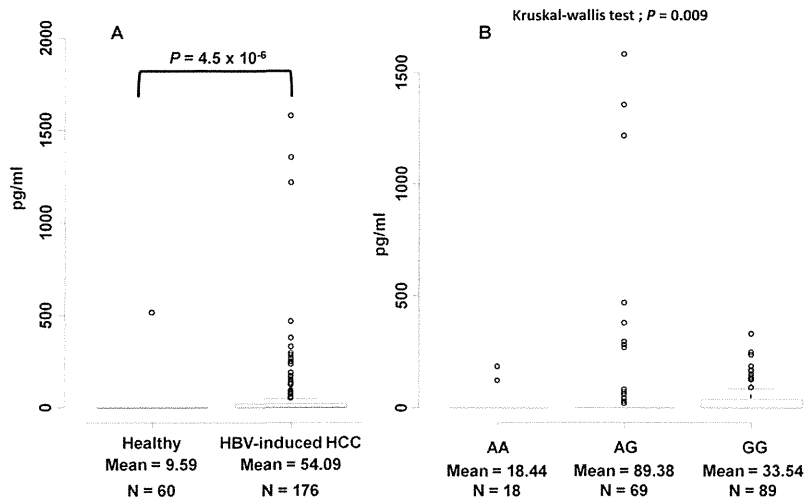

Figure 1. Soluble MICA levels are associated with HBV-related HCC. (A) Correlation between soluble MICA levels and HBV-induced HCC subjects. The y-axis displays the concentration of soluble MICA in pg/ml. The number of independent samples tested in each group is shown in the x-axis. Each group is shown as a box plot and the mean values are shown in the x-axis. The difference between two groups is tested by Wilcoxon rank test. The box plots are plotted using default settings in R. (B) Correlation between soluble MICA levels and rs2596542 genotype in HBV-positive HCC subjects. The x-axis shows the genotypes at rs2596542 and y-axis display the concentration of soluble MICA in pg/ml. Each group is shown as a box plot. $P = 0.027$ and 0.013 for AA vs. GG and AA vs. AG, respectively. The association between genotypes and sMICA levels was tested by Kruskal-wallis test, whereas the difference in the sMICA levels between AA and GG is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
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considered as a stress marker of gastrointestinal epithelial cells because of its induced expression by several external stimuli such as heat, DNA damage, and viral infections [31,32,33,34]. Here, we examined the association of rs2596542 and serum sMICA levels with HBV-induced HCC. Like in HCV-induced HCC [14], our results from ELISA revealed a significantly higher proportion

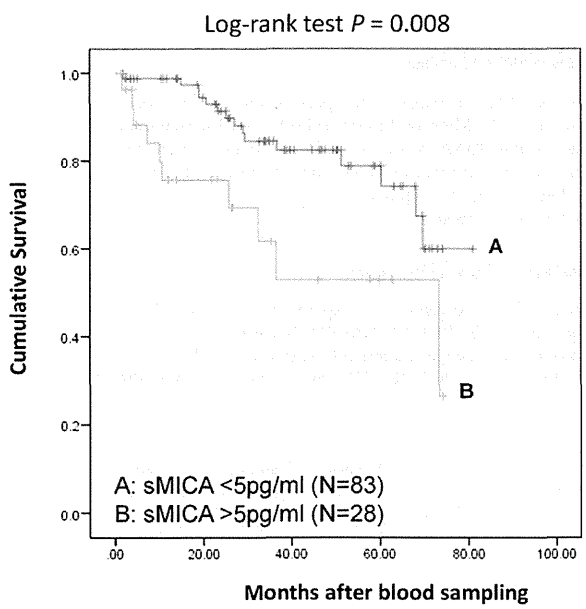

Figure 2. Kaplan-Meier curves of the patients with HBV-induced HCC. The patients were divided into two groups according to their sMICA concentration (high: >5 pg/ml and low: =<5 pg/ml). Statistical difference was analyzed by log-rank test. The y-axis shows the cumulative survival probability and x-axis display the months of the patients survival after blood sampling.
doi:10.1371/journal.pone.0044743.g002

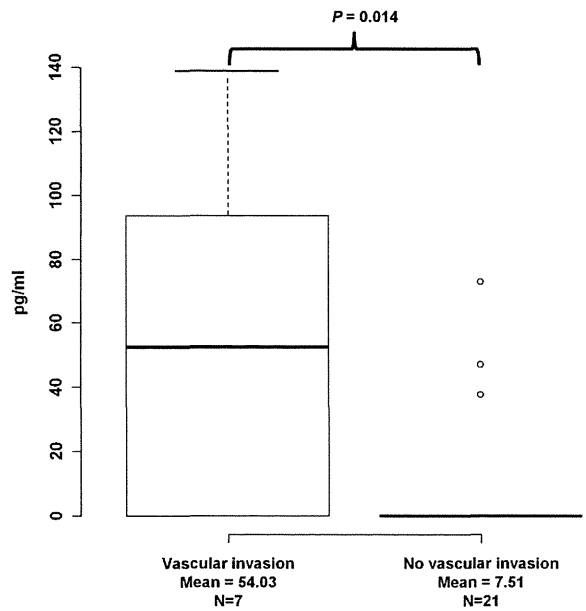

Figure 3. Correlation between soluble MICA levels and vascular invasion in HBV-induced HCC subjects. The y-axis displays the concentration of soluble MICA in pg/ml. The number of independent samples tested in each group is shown in the x-axis. Each group is shown as a box plot and the mean values are shown in the x-axis. The difference between two groups is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
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of high serum sMICA cases (nearly 30%) in the HBV-induced HCC group, compared to non-HBV individuals (1.7%). Moreover, the serum sMICA level was significantly associated with rs2596542, but not with the copy number differences of the VNTR locus, as concordant with our previous report [14].

Several studies have already indicated the roles of sMICA as prognostic markers for different types of malignant diseases [17,18,19,20]. Therefore, it is of medical importance to test whether serum sMICA levels can be used as a prognostic marker for patients with HCC. To our best knowledge, this is the first study to demonstrate the prognostic potential of sMICA for HBV-positive HCC patients; we found 19.3 months of improvement in survival among patients carrying less than 5 pg/ml of serum sMICA, compared to those having more than 5 pg/ml.

On the contrary, we found no significant correlation between sMICA levels and the prognosis of HCV-induced HCC cases. These opposite effects of *MICA* variation could be explained by the following mechanism. The individuals who carry the G allele would express high levels of membrane-bound MICA upon HCV infection and thus lead to the activation of immune cells against virus infected cells. On one hand, HBV infection results in increased expression of membrane-bound MICA as well as MMPs through viral protein HBx [35], which would result in the elevation of sMICA and the reduction of membrane-bound MICA. Since sMICA could block CD8+T cells, NK-CTL, and NK cells, higher sMICA would cause the inactivation of immune surveillance system against HBV infected cells. In other words, HBV may use this strategy to evade immune response and hence, higher levels of sMICA could be associated with lower survival rate among HBV-associated HCC. On the other hand, since HCV is not known to induce the cleavage of membrane bound MICA, individuals with low level membrane bound MICA expression (carriers of rs2596542-allele A) could be inherently susceptible for HCV-induced HCC. Thus, HBx-medicated induction of MMPs could partially explain the intriguing contradictory effect of MICA between HBV-induced HCC and HCV-induced HCC. Since we observed significant correlation of sMICA levels with vascular invasion, it may be the case that high levels of sMICA cause poor prognosis of HBV-related HCC cases by making tumors more aggressive and invasive. However it is important in future to determine the ratio of membrane-bound MICA to sMICA in case of HCV- and HBV-related HCC.

Interestingly, the immune therapy against melanoma patients induced the production of auto-antibodies against MICA [36]. Anti-MICA antibodies would exert antitumor effects through antibody-dependent cellular cytotoxicity against cells expressing membrane-bound MICA and/or activation of NK cells by inhibiting the sMICA-NKG2D interaction. However, further studies are necessary, using well-defined HBV-related HCC cohort, to investigate whether sMICA levels could be included as an additional factor to predict the survival rate among HBV-related HCC subjects. Taken together, our results indicate the potential of *MICA* variant and sMICA as prognostic biomarkers. Thus, MICA could be a useful therapeutic target for HBV-induced HCC.

Supporting Information

Figure S1 MICA repeat genotyping using capillary-based method. The alleles are annotated using GeneMapper software based on the size of the PCR product (185 bp = A4 allele, 188 bp = A5, 189 bp = A5.1, 191 bp = A6 and 200 bp = A9). The inset at the base of each peak shows the size of the PCR product with corresponding allele call by the software. The figure display all observed heterozygotes at A5.1 allele.
(TIF)

Figure S2 MICA VNTR alleles are not associated with soluble MICA levels. Each group is shown as a box plot. The difference in the sMICA values among each group is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
(TIF)

Figure S3 Kaplan-Meier curves of the patients with HCV-induced HCC. The patients were divided into two groups according to their sMICA concentration (<5 pg/ml or >5 pg/ml). Statistical difference was analyzed by log-rank test. The y-axis shows the cumulative survival probability and x-axis display the months of the patients' survival after blood sampling.
(TIF)

Table S1 Clinical parameters of HBV-related HCC patients available for prognostic analyses.
(XLS)

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Author Contributions

Conceived and designed the experiments: VK KM YN. Performed the experiments: VK PHL YU HM ZD. Analyzed the data: VK PHL CT RM. *Contributed reagents/materials/analysis tools:* YN NK AT MK HS KT YT MS MM RT MO KK NK. Wrote the paper: VK PHL KM YN.

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Novel Evidence of HBV Recombination in Family Cluster Infections in Western China

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Abstract

Two hepatitis B virus (HBV) C/D recombinants were isolated from western China. No direct evidence indicates that these new viruses arose as a result of recombination between genotype C and D or a result of convergence. In this study, we search for evidence of intra-individual recombination in the family cluster cases with co-circulation of genotype C, D and C/D recombinants. We studied 68 individuals from 15 families with HBV infections in 2006, identified individuals with mixed HBV genotype co-infections by restriction fragment length polymorphism and proceeded with cloning and DNA sequencing. Recombination signals were detected by RDP3 software and confirmed by split phylogenetic trees. Families with mixed HBV genotype co-infections were resampled in 2007. Three of 15 families had individuals with different HBV genotype co-infections in 2006. One individual (Y2) had a triple infection of HBV genotype C, D and C/D recombinant in 2006, but only genotype D in 2007. Further clonal analysis of this patient indicated that the C/D recombinant was not identical to previously isolated CD1 or CD2, but many novel recombinants with C2, D1 and CD1 were simultaneously found. All parental strains could recombine with each other to form new recombinant in this patient. This indicates that the detectable mixed infection and recombination have a limited time window. Also, as the recombinant nature of HBV precludes the possibility of a simple phylogenetic taxonomy, a new standard may be required for classifying HBV sequences.

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Introduction

Not all viruses are equally prone to recombination. Recombination has not been detected in several viruses despite repeated searches [1]. Whether recombination does or does not exist is important for understanding the evolution and replication mechanism of a specific kind of virus. Hepatitis B virus (HBV), a major human pathogen, has been classified into 10 genotypes and several sub-genotypes [2,3]. Many sub-genotypes were identified by polygenetic analysis as recombinants. But there is no direct evidence to indicate that these subgenotypes arose as a result of recombination or perhaps a result of convergence.

Coinfection with different HBV genotype strains is a prerequisite for recombination. As more than one genotype is predominant in most of the geographic regions, coinfection between the predominating HBV genotypes is not a rare finding, especially for B and C, or A and D. The prevalence of mixed HBV genotype infections has been reported using varied genotyping methods [4,5,6].

Our previous study found two kinds of HBV C/D recombinants in northwest China [7]. In a further study of ethnic groups of five provinces, we confirmed the geographic and ethnic distribution of the HBV C/D recombinant in northwest China [8], and found that family-cluster HBV infections were common in these endemic areas. We hypothesize that infected members of HBV family clusters would gain exposure to various genotypes through marriage, while at the same time; competent strains would be selected through vertical transmission. It would be useful to observe the mixed infection in family-cluster cases, especially in patients infected with C/D recombinants.

The aim of this study was to evaluate the possibility of recombination between two HBV genotypes within an individual by finding cluster-infected families in which individual members were infected with different HBV genotypes. We would then look for individuals within these families with multiple-genotypes that were likely to have been obtained from other family members as a result of vertical or horizontal transmission. Novel viral genomes within an individual with a multiple genotype infection that were mosaics of the known viral genotypes in the family, but not present in any of the other family members, would be consistent with the hypothesis that they arose within the individual with multiple genotype infections.

Methods

Subjects

We enrolled 68 patients with a chronic HBV infection from 15 families. All the families were from a district located at the boundary of Gansu and Qinghai provinces, where the prevalence of genotype C2, D1 and C/D recombinant HBV were known to be high [8]. The families were initially identified with cluster HBV infection in an epidemiological survey in 2002. Sixty-eight individuals were sampled in June 2006 and December 2007 for the purpose of assigning HBV genotypes to chronically infected individuals and finding individuals with multiple HBV genotype co-infections. None of the patients received anti-viral therapy or immunosuppressant drugs. A written, informed consent was obtained from each family, and the study protocol was approved by the Southern Medical University Ethics Committee.

HBV DNA Extraction and HBV Genotyping

HBV DNA was extracted from 400 μL of serum by QIAamp UltraSens Virus Kit (Qiagen GmbH, Germany), then re-suspended in 50 μL water and stored at −20°C until analysis. HBV genotypes, including C/D recombinant, were initially assigned using the PCR based restriction fragment length polymorphism (RFLP) methods described previously [9], [8].

Cloning of Mixed Infection Samples

For samples with mixed genotype infections, PCR cover HBV S gene (nt136-1110) was performed using the primers and thermocycling conditions described by Sugauchi et al [10]. For samples needing further recombination analysis, PCR was performed using the primers and thermocycling conditions described by Günther to obtain full-length HBV genome [11]. Alternatively, a nested PCR was used to produce two overlapping fragments in subjects with low HBV DNA levels as described by Sugauchi et al [12]. The spanning of fragment A cover nucleotides 2813 to 1824, and fragment B included nucleotides 1821 to 237. LA-Taq (TAKARA, Japan) and high-fidelity polymerase COD-FX (TOYOBO, Japan) were used to produce amplimers for cloning and direct sequencing respectively. Finally, Fragment C (HBV nt56-nt1824) was obtained from a PCR amplification of Y2 HBV-DNA to which an aliquot of genotype B HBV-DNA had been added. The purpose of this experiment with in-tube control of genotype B was to determine if the recombinant clones were being generated during the PCR amplification. PCR products were gel-purified and cloned into the PMD19-T vector (TAKARA, Japan) according to the manufacturer's instructions, and used to transform JM109 competent cells (TAKARA, Japan). A minimum of 15 clones were sequenced from subjects with a mixed-strain infection and three clones were sequenced from family members with a single-strain infection. All sequencing of clones and PCR products was performed by Invitrogen Ltd. (Shanghai, China).

Phylogenetic and Recombination Analysis

Genotypes of clones were determined by phylogenetic tree analysis and recombination analysis. The sequences were assembled using SeqMan II software (DNAStar Inc.). Sequence alignments were performed using ClustalW and confirmed by visual inspection. Phylogenetic trees were constructed by the neighbour-joining (NJ) method (Saitou & Nei, 1987). To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. A phylogenetic tree analysis of HBV strains isolated from the mixed infection family was compared with reference strains from GenBank. Accession numbers are indicated on the tree. Bootstrap values are shown along each main branch. The lengths of the horizontal bars indicate the number of nucleotide substitutions per site. The regions included in the analysis were the same with fragment A, B and C or a little shorter. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura, Peterson, Stecher, Nei, and Kumar 2011).

Recombination signals were initially detected by RDP3.β.4 software [13,14]. Bootscan, Geneconv and Siscan were used. The highest acceptable P-value was 0.05. Bootscan and Siscan window sizes were 300 bp, step size was 30, replicates for 100 times. A genotype F sequence (GenBank accession numbers is X75658 and X75663) was used as external reference. The precise map of recombination was determined by split phylogenetic tree and alignment. Split phylogenetic trees were constructed by the method same as above. In alignment, each clone was compared to reference C2, D1 and CD1 consensus sequences. We then inspected the alignments to determine the identical crossover sequences around the breakpoint within which the recombination occurred.

Accession Number of the Sequences

GenBank accession number of reference sequences of HBV genotype C2, D1, CD1 and CD2 are indicated in phylogenetic tree. Accession Numbers of Y2 clones are JX036326-JX036359.

Results

Mixed-genotype Infections in HBV Cluster Families

Different HBV genotypes were found in three families among 15 families. The flow of participants in the study and family trees of families with mixed genotypes/subgenotypes of HBV infection are shown (Figure 1).

Family V had infected members across two generations and two genotypes: In 2006, the mother (V1W) and daughter (V2F) were infected with subgenotype D1 while the son (V2M) had a CD1 recombinant. In 2007, the daughter (V2F) had subgenotype D1 while other family members had HBV DNA levels below the detection limit of the nested PCR assay.

Family Q had infected members across three generations and two genotypes/subgenotypes. In 2006, the grandmother (Q1W) and grandson (Q3M) were infected with CD1 recombinant while father (Q2) and granddaughter (Q3F) had mixed infections of genotype C2 and CD1 recombinants. In 2007, the same genotypes were detected in all family members except that the granddaughter (Q3F) had an HBV DNA level below the detection limit of the nested PCR assay.

Family Y had affected members across three generations and three genotypes/subgenotypes. In 2006, the grandfather of family Y (Y1) was infected with genotype C2 while grandmother (Y1W) had mixed infections of CD1 and C2. Mother (Y2W) and granddaughter (Y3F) were infected with the CD1 recombinant. Father (Y2) had triplicate infections of genotype C2, D1 and CD recombinant. Grandson's (Y3M) serum was unavailable. In 2007, the grandfather (Y1) and mother (Y2W) had HBV DNA levels below the detection limit while the grandmother (Y1W) and granddaughter (Y3F) had genotype CD1. Father (Y2) and grandson (Y3M) had genotype D1.

Phylogenetic Analysis of Family Y, Family Q and Family V

A phylogenetic tree constructed from HBV nt 36-1110 from the clones of family Y is given (Figure 2A). The clones (dotted) of family Y exhibits three clusters on genotype C2, D1 and CD1.

The phylogenetic tree construct from HBV nt136-1110 from the clones of families Q and V is given (Figure 2B). The clones of

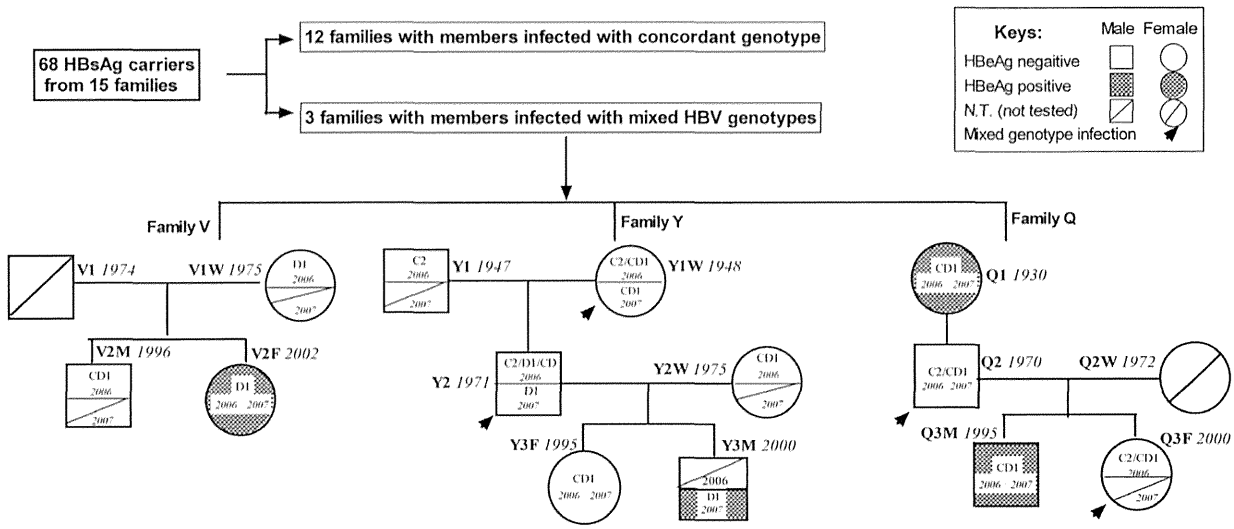

Figure 1. Flow of participants in the study and family trees of family with mixed genotypes/subgenotypes HBV infection. Circles and rectangles correspond to female and male individuals, respectively. Family name and birth date of the patients are indicated beside the circles and rectangles. Subgenotype and the year of blood sampling are indicated inside the circles and rectangles. Family V with affected members across two generations and two genotypes/subgenotypes. Family Q with affected members across three generations and two genotypes/subgenotypes. Family Y with affected members across three generations and three genotypes/subgenotypes. Specially, father (Y2) of family Y with triplicate infection of genotype C, D and CD recombinant in 2006. N.T: Not tested for HBV DNA level below the detection limit of the nested PCR assay or no serum was available.
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family Q (indicated by black dots) exhibit two clusters of subgenotypes C2, and CD1. The clones (indicated by black triangles) from family V exhibit two clusters of subgenotypes D1 and CD1.

A phylogenetic tree constructed from HBV nt 36-1110 of novel recombinants clones of Y2 is given in Figure 2C. The dotted clones are from Y2. The topology of phylogenetic tree with recombinants is totally different from typical trees. Recombinant sequences blurred the typical branch,in other words, blurred the typical genotype.

Recombination and Crossover Analysis of Quasi-species of Y2

Results of recombination analysis of Y2 clones are as bellow: Three kinds of analytical methods certificated the same recombination map. The initial pictures of the three methods were all provided as supplemental figures. Recombination events detected by RDP software are shown in Figure S1, S2, and S3. Split phylogenetic trees constructed by MEGA software are shown in Figure S4, S5, and S6, (clone number and fragment used to construct tree are indicated beside each tree). Sequence alignments are shown in Figure S7, S8, and S9.

The region where recombination breakpoints had the highest probabilities was recognized as crossover region, which is a region that one parental genotype switches to another. Upstream sequence of crossover region will have specific mutation of one genotype but with no specific mutation of another, downstream just opposite. At the same time, these two genotypes should share same sequence at crossover region. We indicated the crossover region in direct alignment by black bars in Figure S3 initially and marked it in recombination map by colorful bars in Figure 3A and black bars in Figure 3B. The clonal sequences of 2006 showed 17 unique crossover regions in fragments A, B and C. We could not identify any common motif within these sequences that might suggest a common mechanism for crossovers in the HBV. The size of switch region share the same sequence are different in different strains, from 6–174 bp (6 bp for Y2M-2 clone in Figure S7 and 174 bp for Y2M-29 clone in Figure S8).

To illustrate the recombination map in a simple way. An abbreviated alignment of fragment A, B and C are shown in Figure 3B. Green and pink bars indicated the genotype C2 and D1 respectively. Black bars showed the crossover region. The aligned sequences provide a snapshot of the recombinant HBV strains. Genotype C2, D1 and CD1 recombinant clones of Y2 were all used as parental sequences to recombine with each other to form new recombinants. A series of novel recombinants were found in three fragments.

In 15 clones of fragment A, there were five genotype C (Y2-6,9,13,14,15,); two genotype D (Y2-11,12); one CD1 (Y2-10) and seven novel different C/D recombination (Y2-1,2,4,7,8,3,5).

In 16 clones of fragment B, there were four genotype C (Y2-23,71,78,75); seven genotype D (Y2-25, 27,79,76,72,22,210); one CD1 (Y2-29) and four novel C/D recombinants (Y2-212,21 73,77).

Of the 56 clones of fragment C(in which genotype B HBVDNA were added as an in-tube control to exclude the recombination by PCR procedure), there were 32 pure genotype B clones; nine genotype C clones(Y2-B10,B5,B8,B9,B13,B16.B17.B18,B24); five genotype D clones(Y2-B22,B3,B4,B21,B23), two CD1 clones (Y2-B1,B11) and eight novel C/D recombinants (Y2-B6,B7,B14,B15,B19,B2,B12,B20). No recombinants of genotype B were found.

Discussion

Recombination is one of the major mechanisms contributing to the evolution of retroviruses [15]. Since the HBV has a reverse transcription step in its life cycle, it is conceivable that recombination also contributes to diversity in HBV genomes. Although just four cases were observed with mixed genotype

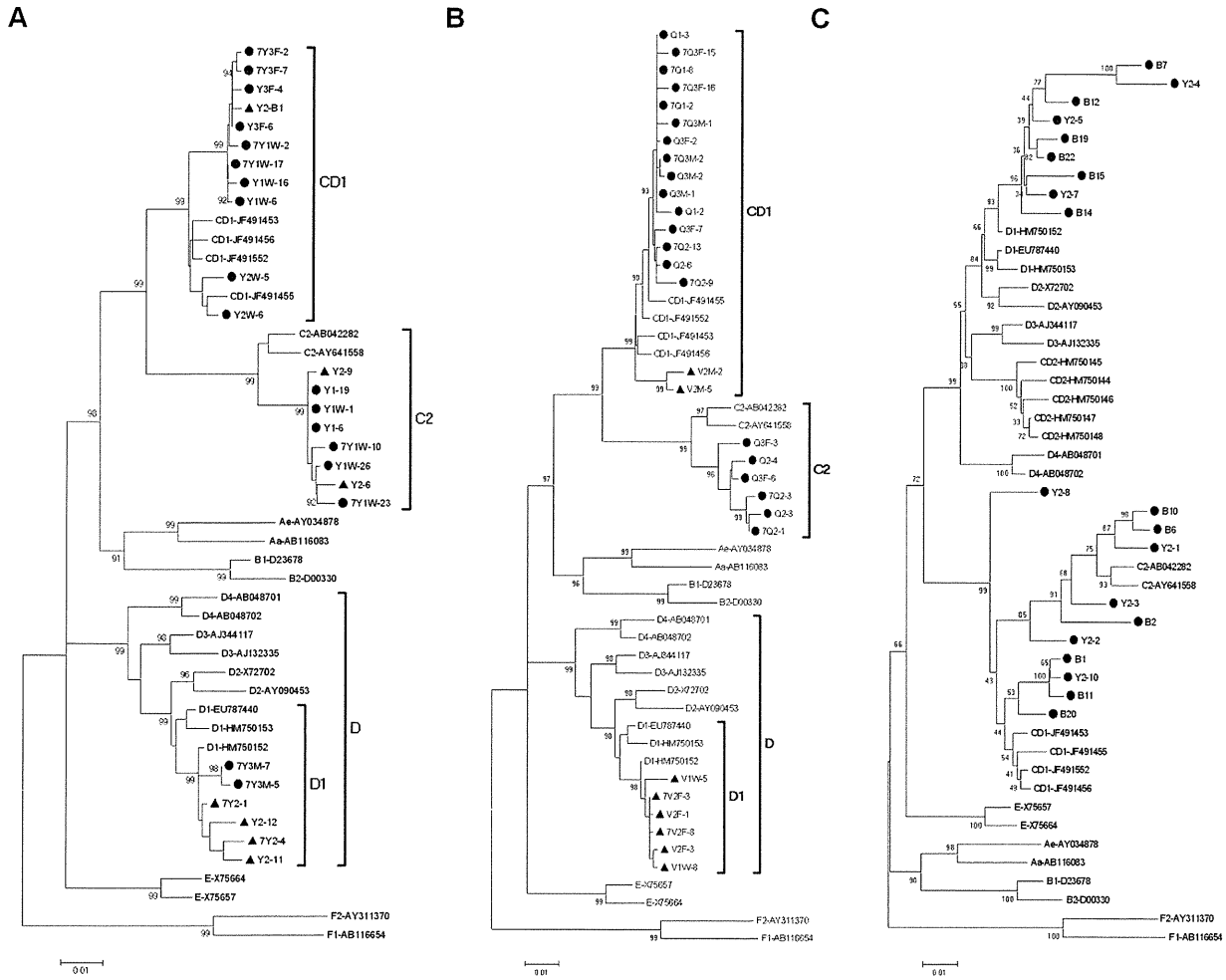

Figure 2. Phylogenetic tree construct by HBV nt 136-1110. (**A**) clones of family Y. Solid dots indicate the clones from Y1,Y1W,Y2W,Y3F and Y3M; Solid triangles indicate the clones from Y2. Family names starting with number 7 means the samples collected in 2007 otherwise in 2006. Novel recombinants of Y2 were excluded from the phylogenetic tree. (**B**) clones of family Q and family V. Solid dots indicate the clones from family Q; Solid triangles indicate the clones from family V. A family name starting with number 7 means the samples collected in 2007, otherwise, in 2006. (**C**) Novel recombinant clones of Y2. Solid dots indicate the clones from Y2.
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infections, we obtained a snapshot of naturally occurring HBV recombinants generated in the absence of selection and after selection. Our result showed direct evidence of HBV recombination, with new information of recombining crossovers compared with similar studies [16,17,18,19].

The recombination analysis of Y2 quasi-species showed variable types of recombinant between genotype C2, D1 and CD1 in 2006. Some studies show that hotspots of recombination most on the boundary of ORFs [12,20]. Our results showed that two or more strains of HBV can recombine with each other at any region along the genome. Crossover regions can be hundreds or just several base pairs, The length of crossover region is depends on the location of it on HBV genome. If it is located in a conserved HBV region, for another word, where many different genotypes share the same sequence, the length of crossover region may be long. If it is located in a non-conserved region, it may be very short. At the same time, we found that the crossover region distributed totally at random on HBV genome. Consistent with our results, *in vitro* evidence showed the initial recombination events in a laboratory system of MHV were almost entirely randomly distributed along the sequence [21]. It was only after passage through cell culture, with the opportunity for selection to remove less fit variants, that crossover sites became "localized" to just a small area of the region examined. Crucially, they also suggested initial products of recombination may go undetected because of the action of strong purifying selection which will remove new, deleterious combinations of mutations. The conclusion is therefore an interpretation for the genotype change of Y2. The Y2 presented multiple strain infections of C2/D1/CD1 and many new recombinants with no obvious dominant genotype strain in 2006. After 18 months, however, all the type C2 and CD recombinant strains disappeared while the D strain became dominant. A similar case of mixed HBV genotype infection in which one genotype was lost and another prevailed was previously described in patients with HBeAg seroconversion [4,22].

Epidemiologically, HBV genotype CD1 and C2 are the most common strains in ethnic minorities of northwest China with CD2 and D1 as minor strains. Precise mapping of recombination