COMPARISON OF HBV RIBONUCLEASE H DOMAIN IN NAÏVE AND DRUG RESISTANT PATIENTS

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Abstract. Nucleotide or nucleoside analog (NA) drug resistance has increasingly become a problem in HBV treatment. Due to the similarity between HBV polymerase and HIV-1 reverse transcriptase, knowledge obtained from HIV research might be applied to the treatment of HBV infection. A previous study has shown that HIV-1 ribonuclease H (RNase H) mutation may contribute to nucleoside reverse transcriptase inhibitor (NRTI) resistance. Therefore, we hypothesized that it might be possible to have a mutation in the HBV RNase H domain of HBV NA drug resistant patients. A one-year cross-sectional study was conducted at a single university hospital. Serum samples were collected from HBV infection treatment naïve and suspected HBV NA drug resistant patients. To confirm HBV NA drug resistance, genotype specific resistance was examined. The HBV genotype and RNase H domain were sequenced and compared. In total, 37 HBV-infected patients were finally analyzed. Of these, 24 were considered sensitive to the drug and 13 resistant, as determined by the genotypic resistance method. Comparison between the two groups showed they had comparable baseline characteristics; no mutation in the HBV RNase H domain was detected. Possibly due to the small sample size, no significant mutations were found in the HBV RNase H domain of either group of HBV-infected patients. Further research of a larger patient group is needed to confirm these initial findings.

Key words: hepatitis B virus, ribonuclease H domain, drug resistiant, mutation, nucleos(t)ide analog drug

INTRODUCTION

Hepatitis B virus (HBV) infection is a major public health problems infecting an estimated 350 million patients worldwide (Lee, 1997; Pawlotsky *et al*, 2008). Current anti-HBV therapy can be divided into 2 groups: interferon and nucleotide or nucleoside analogs (NA). Five NA have been approved to treat HBV infection: lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir (TDF). NA are more advantageous than interferon due to their ease of administration and scarcity of side effects. However, drug resistance has become a major drawback. Mutations in the reverse transcriptase domain of HBV polymerase accounts for the mechanism of antiviral resistance (Pawlotsky *et al*, 2008).

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HBV polymerase consists of 4 subunits: a terminal protein, spacer, reverse transcriptase and ribonuclease H (RNase H) domain, which degrades pregenomic RNA that hybridizes with the minus strand DNA during viral replication (Ghany and Liang, 2007).

The similarity between HBV polymerase and HIV reverse transcriptase has been noted in several studies (Bartholomeusz *et al*, 2004). The HIV-1 RNase H domain is important because of its connection with new anti-viral therapy and the association of RNase H with antiretroviral drug resistance (Schultz and Champoux, 2008). Roquebert *et al* (2007) compared the HIV-1 RNase H domain between HBV infection treatment naïve and nucleoside reverse transcriptase inhibitor (NRTIs) resistant patients and found 4 positions in this domain which mutated more frequently in HIV drug resistant patients than treatment naïve patients. They concluded the RNase H domain mutation may play a role in NRTI resistance. Therefore, the aim of this study was to identify mutations in the RNase H domain in HBV NA resistant patients when compared to naïve patients.

MATERIALS AND METHODS

Study design

HBV-infected patients attending King Chulalongkorn Memorial Hospital, Bangkok, Thailand, were recruited into this cross-sectional study. Permission to carry out this study was given by the ethics committee. The study was carried out for one year.

Eligibility criteria

All eligible patients were included in this study. Inclusion criteria were treatment naïve patients defined as patients who had never been exposed to any NA; patients suspected to have drug resistant HBV infection were defined by one of the following (Lok and McMahon, 2007; Lok *et al*, 2007):

1) Viral breakthrough: an increase in HBV DNA ten-fold above the nadir level after achieving virological response during treatment;

2) Viral rebound: an increase in HBV DNA by more than 20,000 IU/ml above pretreatment level after achieving virological response;

3) Primary drug resistance: a decrease in HBV DNA, but not to less than ten-fold the baseline within the first 3 months after initiation of therapy;

4) Biochemical breakthrough: an increase in ALT after decreasing below the normal upper limit. Patients with HBV/HIV co-infection, were excluded from the study due to polymerase structural homology.

HBV DNA quantitative test

Serum HBV DNA viral load was measured by commercially available automated kit (COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, Roche, Branchburg, NJ). The level was expressed in IU/ml.

HBV genome sequencing

Serum samples were taken from suspected HBV drug resistant cases and treatment naïve patients and stored at -20ºC. To confirm HBV antiviral drug resistance, direct sequencing was performed on the HBV polymerase gene amplified from the serum by polymerase chain reaction (PCR) according to the method previously published (Sa-nguanmoo *et al*, 2008). The HBV genotypes and RNase H sequences were also investigated. The reference strain of HBV sequences was referred to the NCBI database under accession no. NC_003977. The resulting HBV genotypes, drug resistance mutation, and RNase H amino acid

Table 1
Demographic data of the patients in this study.

	Treatment naïve group	NA drug mutation	*p*-value
Number	21	13	
Age[a] (years)	45.8 (13.7)	52.5 (15.2)	0.19
Sex			0.36
Male (%)	13 (61.9)	10 (76.9)	
Female (%)	8 (38.1)	3 (21.1)	
HBV genotypes			0.36
B2 (%)	5 (23.8)	5 (38.5)	
C1 (%)	16 (76.2)	8 (61.5)	
HBV DNA[a]			
IU/ml	2,611,683.85	1,735,395.18	0.50
log IU/ml	6.42	6.24	0.14
HBeAg status			0.10
Negative (%)	9 (42.8)	2 (15.4)	
Positive (%)	11 (52.4)	10 (76.9)	
ALT level[a] (IU/l)	110.2 (150.2)	215.2 (496.1)	0.42

[a]Reported as mean values

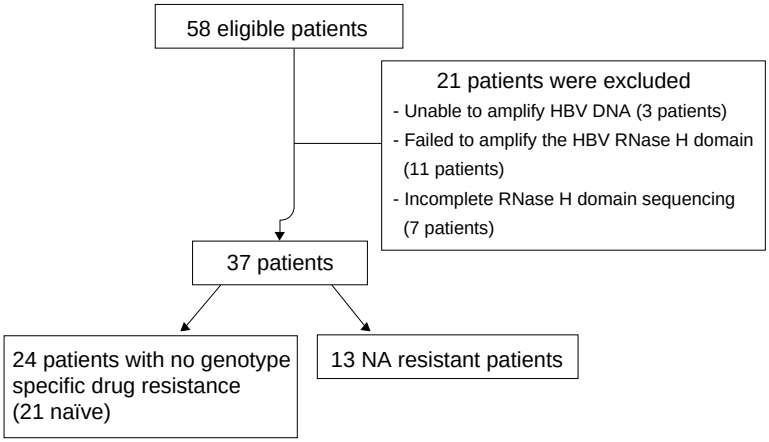

Fig 1–Study design.

sequences were compared between the 2 groups of patients.

Statistical analyses

SPSS version 16.0 was used to calculate the variables. Descriptive data were reported as mean, median, range and percentage. The chi-square test was used to calculate the association between the RNase H domain mutations and NA drug resistance. A p-value < 0.05 was considered as significant.

RESULTS

Fifty-eight patients were eligible for this study. Three and 11 patients were excluded from the study since it was not possible to amplify the HBV DNA and RNase H domain sequences, respectively. Seven samples were discarded because of incomplete RNase H domain sequences. In total, 37 patients were available for final analysis. An outline of the study is shown in Fig 1.

There were 26 males and 11 females with a mean age of 48.95 years (SD 14.68, range 24 to 75). Eleven and 26 patients

Table 2
RNase H domain mutation in HBV-infected patients.

RNase H amino acid position no.	Reference sequence[a]	Treatment naïve group		Drug resistant group		*p*-value
		Substitutions	%	Substitutions	%	
1	R	-	-	L(1)	7.7	0.20
2	S	P(6)	28.6	P(2)	15.4	0.38
12	T	-	-	P(1)	7.7	0.20
19	A	V(1)	4.8	-	-	0.43
20	I	M(2)	9.5	-	-	0.25
23	R	Q(24)	100	Q(13)	100	NA[b]
30	V	L(1), A(1)	9.5	-	-	0.25
31	A	S(5)	23.8	S(5)	38.5	0.36
38	A	E(1)	4.8	-	-	0.43
50	S	-	-	T(1)	7.7	0.20
53	K	R(1)	4.8	N(1)	7.7	0.72
55	I	X(1)	4.8	-	-	0.43
113	H	H(1)	4.8	-	-	0.43
115	P	L(4)	19	E(1),L(1),V(2),W(1)	38.5	0.43
116	F	Y(5)	23.8	Y(5)	38.5	0.36
117	R	-	-	Q(1)	7.7	0.20
122	R	A(1)	4.8	-	-	0.43
124	S	F(1)	4.8	-	-	0.43
125	L	I(1)	4.8	-	-	0.43
128	V	D(5)	23.8	D(4)	30.7	0.66
134	S	F(1)	4.8	F(1)	7.7	0.72
135	H	-	-	R(1)	7.7	0.20
136	L	P(3)	14.3	-	-	0.15
138	D	V(1)	4.8	A(1)	7.7	0.72
147	H	Q(1)	4.8	-	-	0.43
151	R	K(5)	23.8	K(2)	15.4	0.56

[a] Accession no. NA_003977; [b] NA – not available

were identified to harbor B2 and C1 subgenotypes, respectively. The median HBV DNA level was 944,000 IU/ml and the mean HBV DNA level was 5.57 logIU/ml, while the mean ALT level was 190.06 IU/ml. HBeAg was positive in 13 of 31 patients (41.9%) and negative in the remaining 18 patients (58.1%). Although 24 patients (64.9%)did not harbor any drug resistant mutations, 3 of 24 patients were initially suspected to have NA drug resistance and 21 of 24 patients were defined as treatment naïve cases. Thirteen patients (35.1%)were recognized as having NA drug resistance; 11 patient (29.7%), one patient (2.7%) and one patient (2.7%) were identified as having LAM, ADV, and LdT resistance, respectively. There were no patients with ETV or TDF resistance in this study. There were no significant differences demographically between the two groups (Table 1). The 153 amino acids comprising the RNase H domain were compared with the standard sequence previ-

Table 3
Characteristics of nucleos(t)ide analog drug mutation in the patients.

Case	Age (years)	Sex	HBV DNA(IU/ml)	HBV genotype	Drug exposure	Mutation position
1	57	M	3,436,426.11	B2	LAM	L80I, M204I
2	53	M	1,021,271.48	C1	LAM	L80I, M204I
3	63	M	28,197.94	C1	ADV	A181T
4	61	M	3,398,213.06	C1	LAM	L180M, M204I
5	57	F	866.67	C1	LAM	L180M, M204V
6	24	M	28,400.00	C1	LAM	L180M
7	30	M	12,381.79	C1	LAM	V173L, L180M, M204I
8	73	F	28,407.90	C1	LAM	M204I
9	39	M	556.70	B2	LAM	L80V, M204I
10	40	M	8,766.67	B2	LAM	L80V, M204I
11	73	M	3,436,426.12	B2	LAM	M204I
12	54	F	1,292,405.50	C1	LAM	M204V
13	59	M	9,855,714.78	B2	LdT	L80I, M204I

ously described revealing similar polymorphism sequences in both groups; 21/153 (13.7%) in the naïve group and 15/153 (9.8%) in the drug resistant group. The RNase H domain mutations are shown in Table 2. Individual mutations among drug resistant patients were shown in Table 3.

DISCUSSION

This was the first study evaluating possible mutations in the RNase H domain in HBV NA drug resistant patients. No significant mutations were seen between the 2 groups. Roquebert *et al* (2007) discovered mutations in the HIV-1 RNase H domain that might be associated with NRTI resistance, especially a thymidine analog mutation (TAM) which is related to Zidovudine and Stavudine resistance.

We found no data supporting the association between mutations in the HBV RNase H domain and HBV NA drug resistance. This may be explained by the small sample size in our study which resulted in findings not reaching a significant difference. The RNase H domain is considered a highly conserved region; a mutation not compatible with survival (Roquebert *et al,* 2007).

The most common NA drug resistance observed in this study was against LAM, due to the drug's properties which result in a high incidence of drug resistance (Zoulim and Locarnini, 2009). It was the NA drug widely used by physicians in the past and has a good safety profile. The cost of LAM treatment in Thailand is quite low due to generic availability. Because of similarity between types of NA drugs included in this study, we may not be able to unequivocally conclude that HBV RNase H domain mutation is not associated with NA drug resistance in HBV-infected patients.

An additional limitation of this study was the serum samples of NA drug resistant cases should be compared with pretherapy samples in the same patients, which was impossible during the short study. However, it was accepted to com-

pare NA drug resistance with published sequences for the same HBV genotypes (Zoulim and Locarnini, 2009).

The exact cause of the inability to amplify HBV sequences is not known. A possible explanation could be low viral load, since there was some delay between collecting the serum samples and measuring HBV DNA levels. Other probable causes for the inability to amplify HBV sequences include improper storage of serum samples or poor laboratory techniques. Neither scenario is a likely cause in this study. Further research conducted on a larger sample size is needed to confirm these initial findings.

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