JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Serological and molecular analysis on the relationships between type 2 diabetes mellitus and hepatitis B virus infection

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Abstract

Introduction: This study aimed to further analyze the associations between type 2 diabetes mellitus (T2DM) and hepatitis B virus (HBV) infection, and to investigate the relationships between T2DM and the mutations within the HBV major hydrophilic region (MHR).
Methodology: In this cross-sectional study, 3,377 persons (338 T2DM patients and 3,039 non-diabetics) were randomly selected. HBsAg detection was performed by enzyme-linked immunosorbent assay. The HBV MHR was amplified, sequenced, and analyzed by nested PCR.
Results: The seroprevalence of HBsAg was 21.30% in T2DM patients (72/338), which was significantly higher than in non-diabetics (15.53%). Compared to persons without T2DM, the proportion of T2DM patients positive for HBsAg was significantly elevated in males, people > 55 years ($p = 0.039$), and people with a body mass index (BMI) ≥ 24 kg/m^2. Totally, 112 genotype B and 111 genotype C HBV sequences were isolated. No significant difference in HBV genotype distribution was observed between T2DM patients and non-diabetics. Compared to genotype C HBV-infected cases in non-diabetics, the amino acid substitution rates in the MHR were significantly higher in T2DM patients ($p = 0.003$). Moreover, seven HBV strains with stop codon mutations within the HBV *S* gene were identified: three from T2DM patients (5.45%) and four from non-diabetics (2.38%).
Conclusions: In China, T2DM is significantly associated with chronic HBV infections and genotype C HBV MHR mutations. Being males, > 55 years of age, and ≥ 24 kg/m^2 of BMI are the risk factors of HBV infection in T2DM patients.

Key words: type 2 diabetes mellitus; hepatitis B virus; major hydrophilic region; mutation.

J Infect Dev Ctries 2016; 10(8):837-844. doi:10.3855/jidc.7656

(Received 08 September 2015 – Accepted 14 December 2015)

Introduction

Hepatitis B virus (HBV) infection can lead to hepatic fibrosis, cirrhosis, and hepatocellular carcinoma [1,2]. Although safe and effective vaccines for HBV have been available for more than two decades, infection by this organism remains a heavy burden to global public health. Worldwide, more than 2.4 billion people are infected, of which 350 million are chronic carriers of the virus, and over 780,000 people die every year due to HBV-related diseases [3].

Type 2 diabetes mellitus (T2DM) is the most common metabolic disorder in China. Previously, the association between T2DM and HBV infection was studied in different populations [4-7]. Demir *et al*. reported from Turkey that the prevalence of occult HBV infection is higher in diabetics compared with healthy controls [4]. The results from a study conducted in the United States found that the odds ratio (OR) for acute hepatitis B in adults with diabetes was 1.9 (95% confidence interval [CI]: 1.4–1.6) compared with non-diabetics [5]. However, the data from studies in Ethiopia and Nigeria showed no significant difference of HBV infection risk between diabetic patients and controls [6,7]. In addition, by following up a cohort of 500 patients with HBV surface antigen (HBsAg) in Taiwan, Huo *et al*. reported that diabetes was an independent factor associated with liver cirrhosis [8]. Similarly, Huang *et al*. found that patients with chronic HBV infection who developed diabetes were at an increased risk of liver cirrhosis and decompensation [9].

It is well known that disease progression in patients with chronic HBV infection varies widely. Several viral factors, including virus genotype and viral mutations, have been well documented to be strongly associated with disease progression and outcomes [10-13]. However, until now, the association of T2DM with

HBV genome mutations remained unknown. In order to further understand the relationships between T2DM and HBV infection in the Chinese population, the present cross-sectional study was conducted. First, we investigated the difference in HBsAg seroprevalence between T2DM patients and non-T2DM individuals. Second, we analyzed the characteristics of HBV genotype distribution among the surveyed population with and without T2DM. Finally, we further explored the relationships between T2DM and the mutations in the major hydrophilic region (MHR) of the HBV genome, a region highly immunogenic and significantly associated with disease progression.

Methodology

Study population

Between January 2013 and December 2013, 4,191 persons 36–87 years of age were randomly recruited from Suzhou, China, in this cross-sectional study. T2DM status of cases was determined by medical history of a doctor's T2DM diagnosis. Chronic HBV infection was defined as a positive result of HBsAg ≥ six months.

In the present study, 814 people were excluded due to the absence of a history of diabetes diagnosis. Eventually, 1,590 males and 1,787 females (mean age: 52.77 ± 10.10) were included. All of the included subjects had not previously had anti-HBV treatment. None of the persons tested positive for antibodies to HCV, hepatitis D virus, and human immunodeficiency virus. Blood samples were obtained by venipuncture from all individuals and were stored at -80°C until further analysis.

Laboratory testing

The serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were measured with commercial reagents. HBsAg detection was performed by enzyme-linked immunosorbent assay (ELISA) with a commercial ELISA kit (3V Biotechnology, Weifang, China) according to the manufacturer's instruction. The ambiguous results were re-tested and confirmed as positive only if one of the repeats (two-thirds of the total tests) was positive.

HBV DNA isolation, amplification, and quantification

HBV DNA was extracted using a viral RNA/DNA extraction kit, version 5.0 (TaKaRa Biotechnology, Dalian, China) from 200 μL of plasma according to the manufacturer's instructions. A 437-bp region of the *S* gene (region nt220–nt656, reference strain HBV-Chi89, AB073826) was amplified by nested polymerase chain reaction (PCR). Primers DZ-1 (5'-CTAGGACCCCTGCTCGTGTTAC) and DZ-4 (5'-CACTGAACAAATGGCACTAG) were used for the first round of PCR, and primers DZ-2 (5'-GACAAGAATCCTCACAATAC) and DZ-3 (5'-CTGAGGCCCACTCCCATAG) were used for the nested PCR, respectively.

HBV DNA titer was analyzed by real-time PCR with a diagnostic kit for quantification of hepatitis B virus DNA (Kehua Bio-engineering Co., Shanghai, China). The range of the quantification of HBV DNA is 500–10^5 copies/mL.

Sequencing and analysis

The 437-bp PCR products were sequenced by the sequence analyzer ABI 3730 (Applied Biosystems, Foster City, USA) after purification by a gel extraction kit (Tiangen Biotechnology, Beijing, China). The sequences were aligned using the Clustal W program in MegAlign version 3.17 software. For further analysis, the *S* gene sequences were cropped to 396 nucleotides (nt 236–631) and the MHR gene sequences were cropped to 183 bp (nt 449–631) because of the poor quality of some sequences.

Based on reference sequences of known HBV genotypes A–H, the genotypes of isolated HBV were identified using phylogenetic analysis by the neighbor-joining (NJ) algorithm in MEGA version 4.1 software. The reliability of the phylogenetic tree was tested by bootstrap analysis with 1,000 replicates. The Kimura two-parameter model was used to estimate the nucleotide homology of the sequences. HBV reference wild-type sequences of genotype B (AB073826) and genotype C (AF286594) were obtained from GenBank and used to identify nucleotide and amino acid (aa) variability. The partial region of the MHR (99–159 aa of 99–169 aa) was compared with the reference sequence of the same genotype in BioEdit software.

Statistical analysis

Statistical analysis was performed using SAS version 9.2 software (SAS Institute Inc., Cary, USA). The prevalence of nucleotide and amino acid mutations were compared by χ^2 test or Fisher's exact test, as appropriate. An unpaired Student's *t*-test was used for continuous variables. Multivariate analyses using logistic regression were performed to elucidate the relationship between HBsAg seropositivity and diabetes mellitus. All tests were two sided, and differences were considered statistically significant if p values were < 0 05.

Nucleotide sequence accession numbers

The clinical isolates characterized in this study have been deposited in GenBank under accession numbers KP410408–KP410460 (except for KP410422, KP410437, and KP410453) and KP749956–KP750172 (except KP749975–KP749999, KP750034–KP750035, KP750090–KP750091, KP750142, KP750146, KP750154–KP750165, and KP750167).

Ethical approval

This study was approved by the ethical committee of the Centers for Disease Control of Suzhou Industrial Park in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Results

Higher HBsAg detection rate in T2DM patients

A total of 3,377 persons, including 338 diabetes and 3,039 non-diabetes subjects, were selected in the present study based on the above-described criteria. The mean values of ALT, AST, and body mass index (BMI) of the studied population were 20.92 ± 14.85 IU/L, 25.91 ± 10.30 IU/L, and 23.76 ± 3.77 kg/m^2, respectively.

As shown in Table 1, 544 of 3,377 subjects were seropositive for HBsAg. Among of 338 subjects with T2DM, 72 (21.30%) cases carried HBsAg, a proportion which was significantly higher than that in persons without diabetes (15.53%). This difference was significant, and remained so after adjusting for gender and age (OR: 1.54, 95% CI: 1.16–2.04, p = 0.003). In addition, compared to persons without diabetes, the proportion of T2DM patients positive for HBsAg was significantly elevated in males (OR: 1.96, 95% CI: 1.36–2.80, p < 0.001), in people older than 55 years of age (OR: 1.56, 95% CI: 1.02–2.39, p = 0.039), and in persons with a BMI ≥ 24 kg/m^2 (OR: 1.55, 95% CI: 1.07–2.26, p = 0.020).

Phylogenetic analysis of HBV strains isolated from studied populations

HBV DNA detection assays were completed in 72 HBsAg-positive T2DM patients and 472 healthy controls; 55 and 168 strains were detected, respectively. The mean titer of HBV DNA (log_{10} copies/mL) was 3.28 ± 1.22 and 3.20 ± 1.37 in diabetics and non-diabetics. No significant difference was detected between the two groups.

Of 55 HBV strains isolated from T2DM patients, 31 sequences (56.36%) belonged to genotype B, with the nucleotide homology ranging from 96.2% to 100%. The other 24 (43.64%) strains belonged to genotype C, with 91.7%–100% nucleotide homology. Among 168 HBV sequences amplified from non-diabetic controls, 81 (48.21%) strains belonged to genotype B and 87 (51.79%) sequences belonged to genotype C. The nucleotide homology was estimated to be 93.9%–100.0% and 92.4%–100.0% with genotype B and C HBV, respectively. No other genotypes were detected. Moreover, there was no significant difference of

Table 1. Comparison of HBsAg seropositivity between type 2 diabetes mellitus (T2DM) patients and non-diabetics.

Variable		HBsAg		Unadjusted		Adjusted*	
		Positive	Negative	OR (95% CI)	p	OR (95% CI)	p
		n (%)	n (%)				
	Non-diabetics	472 (15.53%)	2,567 (84.47%)	1 (Ref)	0.006	1 (Ref)	**0.003**
	T2DM	72 (21.30%)	266 (78.70%)	1.47 (1.12–1.95)		1.54 (1.16–2.04)	
Gender							
Male	Non-diabetics	217 (15.44%)	1,188 (84.56%)	1 (Ref)	< 0.001	1 (Ref)	**< 0.001**
	T2DM	48 (25.95%)	137 (74.05%)	1.92 (1.34–2.75)		1.96 (1.36–2.80)	
Female	Non-diabetics	255 (15.61%)	1,379 (84.39%)	1 (Ref)	0.979	1 (Ref)	0.727
	T2DM	24 (15.69%)	129 (84.31%)	1.01 (0.64–1.59)		1.09 (0.68–1.72)	
Age							
36–55	Non-diabetics	316 (16.41%)	1,610 (83.59%)	1 (Ref)	0.047	1 (Ref)	0.051
	T2DM	40 (22.22%)	140 (77.78%)	1.46 (1.00–2.11)		1.45 (1.00–2.11)	
56–87	Non-diabetics	156 (14.02%)	957 (85.98%)	1 (Ref)	0.040	1 (Ref)	**0.039**
	T2DM	32 (20.25%)	126 (79.75%)	1.56 (1.02–2.38)		1.56 (1.02–2.39)	
BMI							
< 24 (kg/m^2)	Non-diabetics	283 (15.97%)	1,489 (84.03%)	1 (Ref)	0.067	1 (Ref)	0.058
	T2DM	29 (22.14%)	102 (77.86%)	1.50 (0.97–2.31)		1.53 (0.99–2.36)	
≥ 24 (kg/m^2)	Non-diabetics	189 (14.92%)	1,078 (85.08%)	1 (Ref)	0.033	1 (Ref)	**0.020**
	T2DM	43 (20.77%)	164 (79.23%)	1.50 (1.03–2.16)		1.55 (1.07–2.26)	

* OR was adjusted for age and gender. When compared in different gender groups, the OR was adjusted for age. When compared in different age groups, the OR was adjusted for gender; BMI: body mass index.

genotype B and C virus distribution between these two groups (p = 0.294). The phylogenetic tree is shown in Figure 1.

Stop codon mutations in HBV S gene in T2DM patients

In total, seven genotype C HBV strains with stop codon mutations in the HBV *S* gene were identified: three strains from T2DM patients and four sequences from non-diabetic controls. Among the seven mutated HBV strains, four sequences (one in the T2DM group and three in the control group) showed a change in aa position 69 from TGT (cysteine) to TGA (stop). Two strains from T2DM cases showed a substitution at aa position 61 of HBsAg, due to the TCA-TAA mutation. One sequence of non-diabetics contained a stop codon at position 74 (TGG-TGA) (Figure 2). However, although the rate of stop codon mutations that happened in T2DM patients was about twice that in non-diabetics (5.45% vs 2.38%), a statistical difference was not observed (p = 0.368).

Increased amino acid substitutions in the MHR of genotype C HBV in T2DM patients

A total of 21 of 52 HBV strains (40.38%) in diabetics had 43 aa substitutions in the MHR. Among 164 strains from non-diabetics, 54 (32.93%) virus strains had aa substitution in the MHR, with a total of 98 mutations (Figures 3 and 4). No significant difference was detected between the two groups.

Figure 1. Phylogenetic analysis based on nucleotide sequencing of the 396 bp (nt236–nt631) of the S region. There were 31 strains of genotype B HBV ("▲"), 24 strains of genotype C HBV ("●") from T2DM patients and 81 strains of genotype B HBV ("△"), 87 strains of genotype C HBV ("○") from non-diabetics controls were isolated in the present study. Genotype A (AB194949, AJ309369, AM180623, AY233275, AF090838), genotype B (AB033554, D00329, EF494380, EU579441, FJ562257), genotype C (AB014381, AF330110, AJ74809, AY040627, EF494376), genotype D (AB554016, HE815465, KC875317, X65259, X85254), genotype E (AB091255, AF323631, AJ605025, AP007262, AR488645), genotype F (AB036905, AF223965, AY179735, DQ823086, HE981185), genotype G (AB064310, AF160501, AP007264, DQ207798, EF464098), and genotype H (AB179747, AP007261, AY090454, EF157291, EU498228) were used as reference HBV strains.

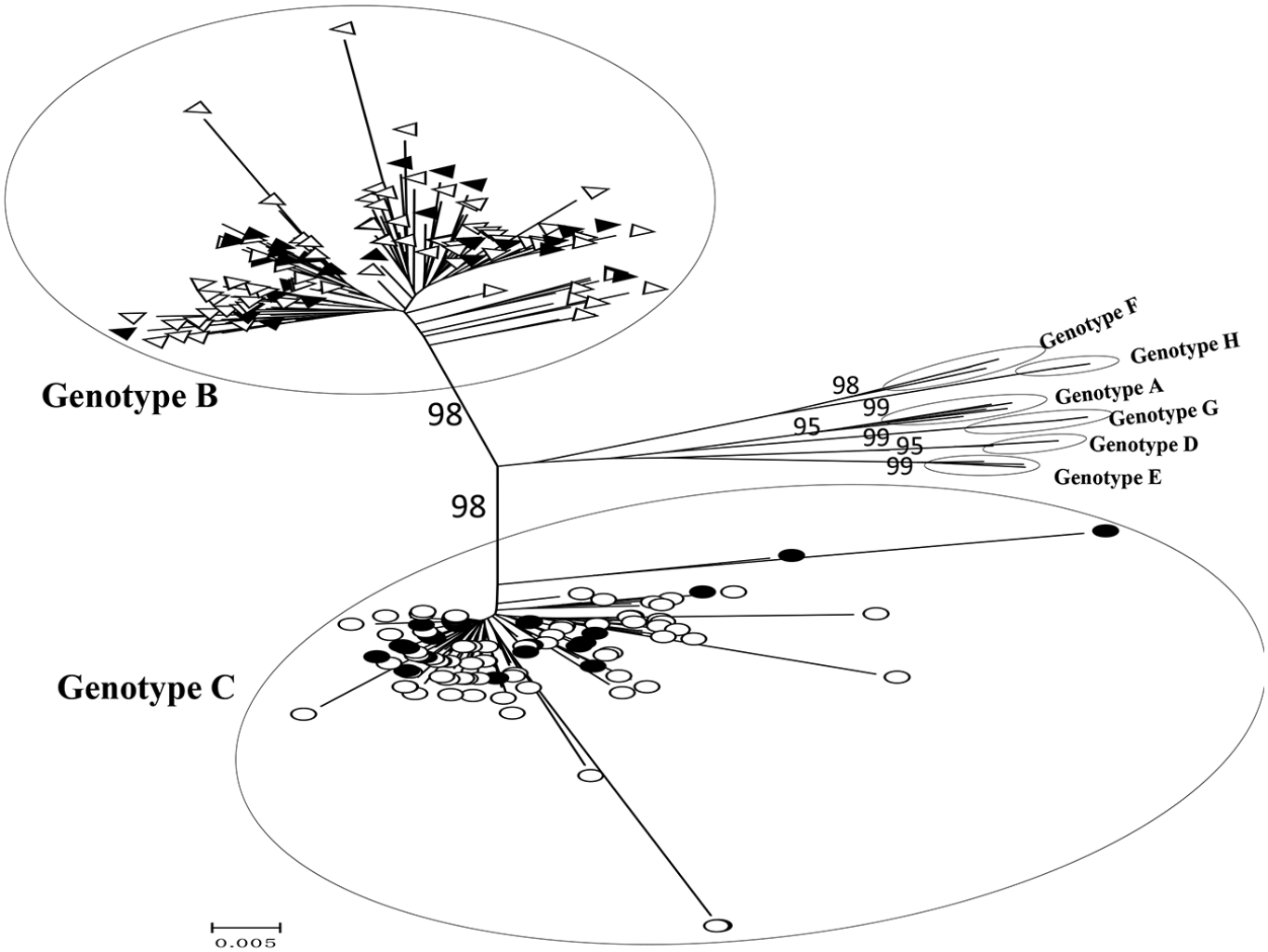

Figure 2. Seven genotype C HBV strains with stop codon mutations in the *S* gene region isolated from this study. Three strains were from T2DM patients and four sequences were from non-diabetic controls. The consensus nucleotide sequence of genotype C was deduced from AF286594.

		58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
		S	N	H	S	P	T	S	C	P	P	I	C	P	G	Y	R	W	M
Wild	AF286594	TCC	AAT	CAC	TCA	CCA	ACC	TCT	TGT	CCT	CCA	ATT	TGT	CCT	GGC	TAT	CGC	TGG	ATG
T2DM	KP749962	TCC	AAT	CAC	TAA*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T2DM	KP749964	TCC	AAT	CAC	TAA*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T2DM	KP750022	TCC	AAT	CAC	TCA	CCA	ACC	TCT	TGT	CCT	CCA	ATT	TGA*	-	-	-	-	-	-
Non-diabetics	KP750080	TCC	AAT	CAC	TCA	CTA	ACC	TCT	TGT	CCT	CCA	ATT	TGA*	-	-	-	-	-	-
Non-diabetics	KP750040	TCC	AAT	CAC	TCA	CCA	ACC	TCT	TGT	CCT	CCA	ATT	TGT	CCT	GGC	TAT	CGC	TGA*	-
Non-diabetics	KP750086	TCC	AAT	CAC	TCA	CCA	ACC	TCT	TGT	CCT	CCA	ATT	TGA*	-	-	-	-	-	-
Non-diabetics	KP750119	TCC	AAT	CAC	TCA	CCA	ACC	TCT	TGT	CCT	CCA	ATT	TGA*	-	-	-	-	-	-

Figure 3. Amino acid substitutions within the major hydrophilic region (MHR) (99–159 aa) in genotype B HBV infected subjects. The consensus amino acid sequence of genotype B and HBV was deduced from AB073826. 31 strains isolated from T2DM patients and 81 sequences amplified from non-diabetics controls are presented in this figure.

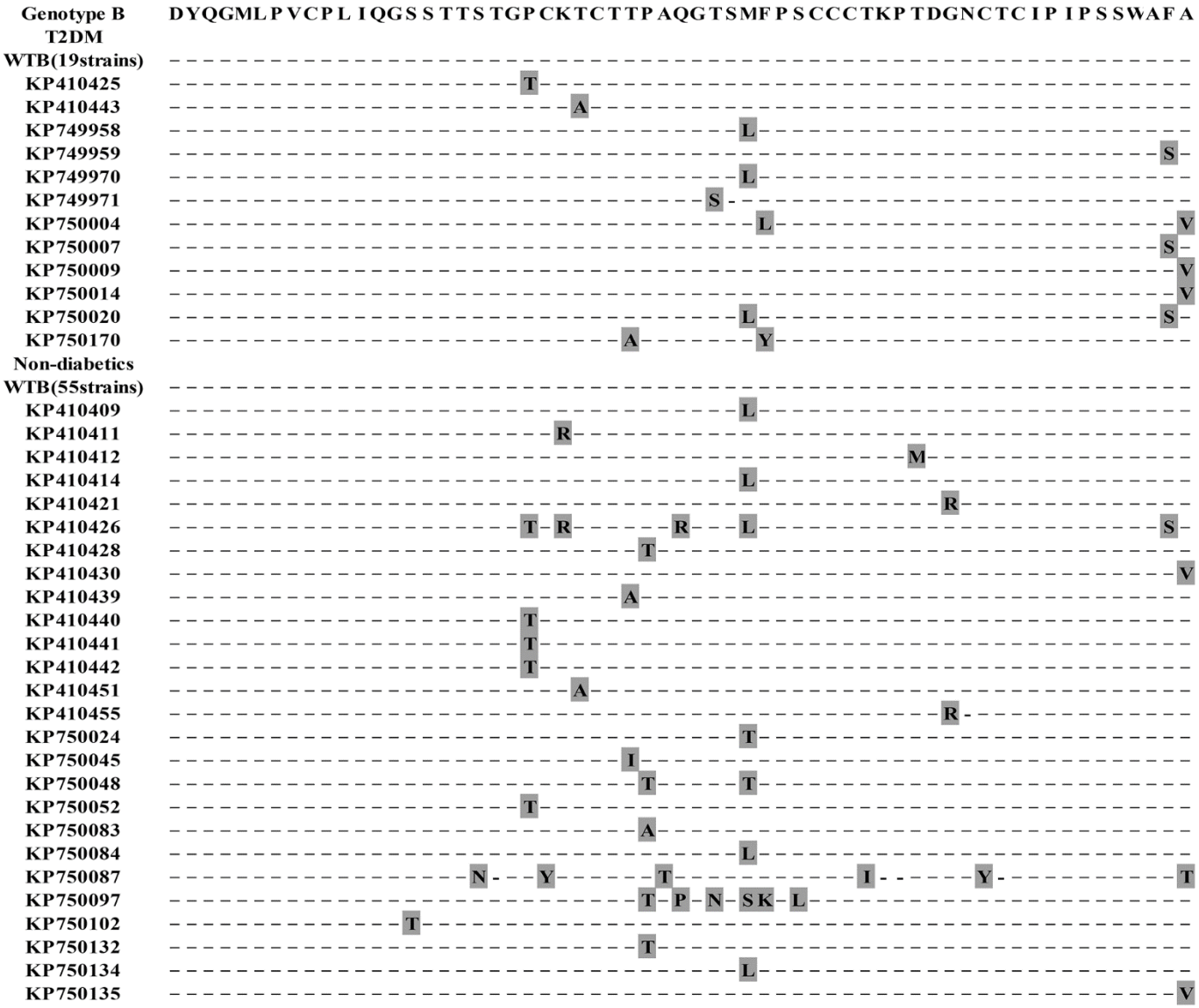

Figure 4. Amino acid substitutions within the major hydrophilic region (MHR) (99–159 aa) in genotype C HBV-infected subjects. The consensus amino acid sequence of genotype C HBV was deduced from AF286594. 24 strains isolated from T2DM patients and 87 sequences amplified from non-diabetics controls are presented in this figure.

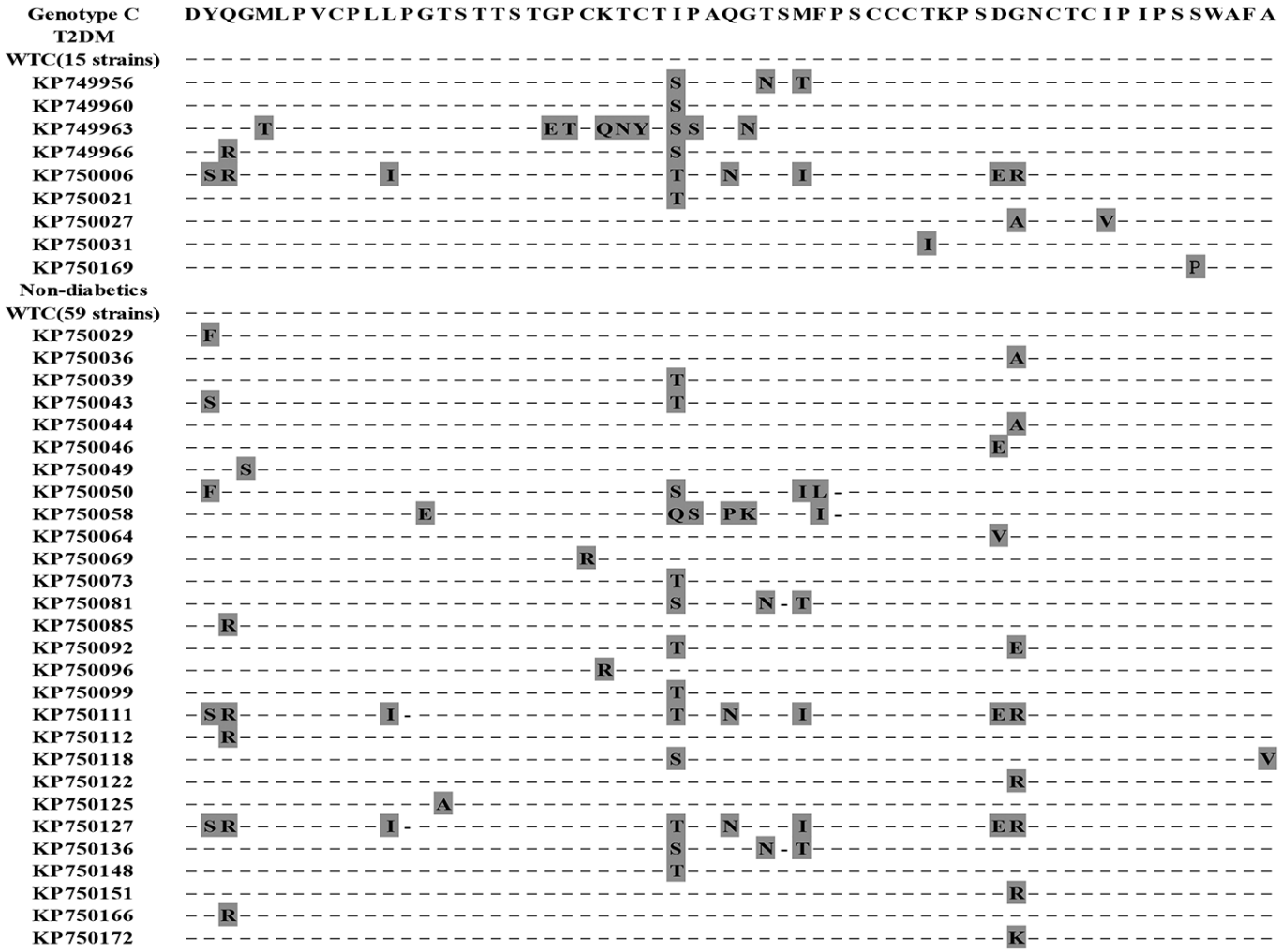

Table 2. Comparison of amino acid substitutions within the major hydrophilic region of HBV isolated from type 2 diabetes mellitus (T2DM) patients and non-diabetics.

Amino acid substitutions	T2DM	Non-diabetics	χ^2	p
Total			**3.22**	**0.073**
Substituted	43 (1.36%)	98 (0.98%)		
Non-substituted	3,129 (98.64%)	9,906 (99.02%)		
Genotype B			**0.02**	**0.881**
Substituted	15 (0.79%)	41 (0.83%)		
Non-substituted	1,876 (99.21%)	4,900 (99.17%)		
Genotype C			**8.69**	**0.003**
Substituted	28 (2.19%)	57 (1.13%)		
Non-substituted	1,253 (97.81%)	5,006 (98.87%)		

Of note, among genotype C HBV isolated from the present study, 21 strains from T2DM cases had a total of 28 aa mutated in the MHR, while 57 substitutions were detected in 83 strains from non-diabetics. There was a significant difference of amino acid substitutions in the MHR between T2DM patients and controls (2.19% vs 1.13%, p = 0.003). However, for the genotype B HBV isolated in this study, no significant difference in amino acid mutations in the MHR was detected between these two groups (Table 2).

Discussion

In the present study, we found that the seropositivity of HBsAg in T2DM patients was significantly higher than those in non-diabetic controls (21.30% vs 15.53%). Additionally, compared to persons without diabetes, the proportion of T2DM patients positive for HBsAg was significantly elevated in males, people older than 55 years of age, and in persons with BMIs ≥ 24 kg/m^2. In a previous study, Li-Ng M *et al*. reported that Asians had higher seropositivity of HBsAg compared to Pacific Islanders (22.5% vs 7.0%), and also that HBV infection was strongly associated with diabetes mellitus among Asian Americans [14]. Taking these findings together, our results suggest that T2DM could be considered to be a risk factor for HBV infection in China.

Genotypes B and C HBV are predominant in China [10,15,16]. Infection with genotype C HBV has been reported to be associated with severe liver disease, such as cirrhosis and hepatocellular carcinoma, when compared with genotype B [17,18]. HBsAg is the major envelope protein of HBV and is encoded by the *S* gene. The MHR is a highly immunogenic region of the *S* gene and is potentially under selective pressure of the immune system [19-21]. Interestingly, we observed that increased amino acid mutations occurred within the MHR in genotype C HBV isolated from T2DM patients when compared to those from non-diabetics. It is well known that mutations within the MHR are the important factors associated with vaccine escape, immunotherapy escape, failure of antiviral therapy, and disease progression [22-24]. Therefore, the present data might partially explain the mechanisms of diabetes on the acceleration of the progression of HBV infection. Moreover, the association between HBV genome mutation and HBV disease progression among T2DM should be further investigated in the future, especially in genotype C HBV-infected T2DM patients.

We found seven HBV strains (three in diabetics and three in non-diabetics) containing stop codon mutations in three different HBsAg positions: four at position 69, two at position 61 and one at position 74. Some previous studies observed that cysteine 69 was highly conserved and essential for HBsAg secretion from infected hepatocytes [25,26]. A stop codon mutation at this position may lead to the accumulation of the prematurely truncated form of HBsAg into the hepatocyte and may induce transactivation of cellular promoters, including those encoding oncogenic proteins. Moreover, this mutation has been associated with antiviral treatment failure [27]. Since the HBV *S* gene is the main target for the specific antibody against HBV, these mutations may result in the escape of the virus from the neutralizing antibody response or the failure of antiviral therapy [19,28,29]. Therefore, the stop codon mutations in the *S* gene should be further investigated in T2DM patients with HBV infection, although the results of the present study did not show a statistical difference between diabetics and non-diabetics.

Conclusions

Our results suggest that T2DM is significantly associated with chronic HBV infection in China. Being male, > 55 years of age, and having a BMI ≥ 24 kg/m^2 are the risk factors of HBV infection in T2DM patients. Moreover, this is the first study to report that T2DM was significantly associated with genotype C HBV MHR mutations. Considering both T2DM and chronic HBV infection are serious public health issues in China, our study contributes to the further understanding of the HBV infection in T2DM patients.

Acknowledgements

We deeply appreciate Dr. Teo Chong-Gee in the division of viral hepatitis, Centers for Disease Control and Prevention (Atlanta, Georgia) for his kind help with this manuscript. This work was supported by the project sponsored by SRF for ROCS, SEM (K513901814), the Soochow University High-level Scientific Research Foundation for the Introduction Talent (Q413900612), Pre-research Foundation for Nature Science of Soochow University (Q313904713), and Research Foundation for the Prevention Medicine in Jiangsu province (Y2012024).

Authors contribution

Z.H. and W.Y. contributed equally to these work.

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Conflict of interests: No conflict of interests is declared.