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Research Article

Hepatitis B amongst Immigrants from Myanmar: Genotypes and their Clinical Relevance[1].

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Shortened title: Hepatitis B Genotypes in Immigrants from Myanmar

ABSTRACT

Hepatitis B virus (HBV) from 76 adult immigrants in Australia from Myanmar was characterised to determine the prevalence of different HBV genotypes and subgenotypes. A mutational analysis was then performed to determine the presence of clinically significant mutations and correlate them to clinical outcomes.

Initial genotyping revealed 68 patients with genotype C (89.5%) and eight patients with genotype B (10.5%). Phylogenetic analysis revealed the large majority of the genotype C infections were of subgenotype C1 (67/68).

Sequencing of the HBV polymerase gene (and overlapping surface gene) revealed no mutations associated with antiviral resistance. HBV surface gene mutations were detected in 10 patients with subgenotype C1. HBV BCP/PC sequencing was obtained for 71/76 (93%) patients. BCP and/or PC mutations were identified in 57/71 (80%) of PCR positive patients.

Treatment had been commenced for 15/76 (18%) patients, a further 26 untreated patients were in a stage of disease where HBV treatment would be considered standard of care.

It was identified that genotype C1 is the predominant sub-genotype in this population. Genotype C is known to be associated with increased risk of development of HCC.

This highlights the need for screening for HCC given the potential for the development of liver cancer. It was also identified that people with HBV were potentially not receiving optimal therapy in a timely fashion.

KEY WORDS: Hepatitis B Virus Genetics, Myanmar, Immigrant

INTRODUCTION

Chronic Hepatitis B virus (CHB) infection affects approximately 248 million people worldwide. [1] CHB prevalence is highest in eastern Asia and Africa [1] and chronic infection leads to death in up to 25% of those infected due to hepatocellular carcinoma (HCC) and liver cirrhosis. [2,3]

In Australia, an estimated 218,000 people (1% of total population) are living with CHB, the majority (56%) of whom were born overseas [4] predominantly from the following regions: Asia/Pacific 38%, Europe 10%, Africa/Middle East 7%, The Americas: 0.8%. People from culturally and linguistically diverse (CALD) backgrounds, particularly Asia-Pacific or Sub-Saharan Africa, are listed as one of the priority populations in the Australian National Hepatitis B Strategy [5] and as such are an important group to focus on for screening and therapy.

Myanmar (Burma) has suffered fifty years of military dictatorship, as well as ongoing civil war. As a result minority groups have fled to refugee camps along the borders of India, Bangladesh and Thailand, as well as to Malaysia. High rates of CHB have been identified amongst immigrants from Myanmar in both Australia (9.7%)[6] and the USA (9.4 %).[7] It is estimated that the seroprevalence of CHB in Myanmar is about 8%, making it an area of high prevalence. [8,9] Almost half of the population of Australians from Myanmar (24000) arrived after 2006, and 80% are under 45 years of age. [10] There are 135 distinct ethnic groups recognised in Myanmar. The largest groups are Burmese (Bamar) (68%), Shan (9%) and Karen (Kayin) (7%). [11] Many of the immigrants from Myanmar living in Australia have come from the Thai-Myanmar border and are of Karen ethnicity. [6]

Many clinical and molecular-based studies in Australia have predominantly focussed on CHB in Chinese and Vietnamese immigrants. These studies have found that genotypes B and C predominate in South East Asia and China. Genotype C is known to be associated with an increased risk of development of HCC. [12] Co-infection with more than one genotype is

possible, and in Asia co-infection with both genotype B and C is common. [13] Some Hepatitis B virus (HBV) recombinants are now endemic in certain geographic regions, and have been assigned their own genotype or subgenotype, most notably the recombination between isolates of HBV genotypes B and C [14] resulting in HBV subgenotype B2, which contains the precore and core genes of an HBV genotype C, and is found throughout Asia. [14]

Within Myanmar genotype C has been identified as the predominant genotype, both amongst local residents and those migrating from Myanmar. [8,15,16] Genotype C is associated with a later seroconversion from Hepatitis B e antigen (HBeAg) positive status to HBeAg negative, anti-HBe positivity and more advanced liver disease. [17] Other studies however, have focused on populations such as from Korea with largely genotype C2, and it is unknown if this is also the case with genotype C1. [18] Basal core promotor (BCP) and/or precore (PC) mutations have been shown to be associated with increased progression to cirrhosis and/or HCC. [19] Routine use of genotyping may assist in the selection of patients for treatment, as has been recommended for Asian patients with genotype B or C virus, and may identify patients at higher risk of progression of liver disease and HCC. [20]

The aim of this study was to characterise HBV isolates from Myanmar-born immigrants living in Victoria, Australia. We aimed to determine the prevalence of different HBV genotypes/subgenotypes, and also perform a mutational analysis to determine the presence of clinically significant mutations which may influence clinical outcomes. This information was correlated with patient clinical information including disease stage and treatment status.

METHODS

This was a retrospective, observational study. Ethics approval was received from the Royal Melbourne Hospital Human Research and Ethics Committee.

Patient Samples

Stored serum was obtained for 84 patients from Myanmar from three separate sites in Victoria - University Hospital, Geelong, Isis Primary Care (Hoppers Crossing) and the Royal Melbourne Hospital hepatitis and travel/refugee clinics. All samples had been sent to the Victorian Infectious Diseases Reference Laboratory (VIDRL) as part of routine clinical care.

Clinical data

Where possible sociodemographic and clinical data were collected retrospectively from medical charts together with pathology results, and were entered into a standardised case report form (CRF). The following sociodemographic parameters were collected: date of birth, gender, ethnicity, country of origin and age. Pathology results included aspartate aminotransferase (AST), alanine amino transferase (ALT), bilirubin, albumin, platelets, HBeAg status and HBV viral load (IU/mL). Information on clinical management (treatment yes/no, drug used for treatment, imaging studies including transient elastography results) was recorded where available. Data were censored on 30th December 2013.

Clinical information was compiled in a separate Excel© (Microsoft, Redmond, Washington, USA) database for further analysis. Disease stage was determined using recognised staging systems. [21,22] These were immune tolerant stage (HBeAg positive, viral load > 20000iu/mL, ALT <45 iu/L), immune clearance (HBeAg positive, viral load > 20000iu/mL, ALT >45 iu/L), immune control (HBeAg negative, viral load < 2000iu/mL, ALT < 45iu/L, and immune escape (HBeAg negative, viral load > 2000iu/mL, ALT >45iu/L). Statistical analysis was performed using STATA SE12© (StataCorp, College Station, Texas, USA). Fisher's exact test was used to compare categorical data of multiple groups. Kruskal Wallis test was used for numerical data comparison, assuming non-normal distribution of the data.

HBV DNA Extraction, PCR and Sequencing

HBV DNA was extracted according to previously published protocols. [23]

To determine genotype/subgenotype, a 893bp fragment of the reverse transcriptase (RT) region of the polymerase gene (and corresponding overlapping region of the surface (S) gene) was amplified and sequenced using primers Seq2 5'-TTG GCC AAA ATT CGC AGT C-3' and 2996 5'-GCG TCA GCA AAC ACT TGG C-3'.To determine the presence of basal core promoter (BCP) and precore (PC) mutations, amplification and sequencing of a 356bp fragment of the BCP/PC region was performed using primers PC5 5'-TCG CAT GGA GAC CAC CGT GA-3' and PC2 5'-GGC AAA AAC GAG AGT AAC TC-3'.

Amplification and sequencing was conducted as per previously published protocols. [23]

HBV consensus sequences for the polymerase and BCP/PC genes were constructed using the DNA sequence analysis program *SeqScape* (ABI Prism, Applied Biosystems, Foster City, CA). HBV genotype and unique HBV mutations were identified using a web-based analysis program, *SeqHepB*, as previously described. [24,25]

Phylogenetic Analysis

The HBV polymerase sequences obtained were compared to a set of published reference sequences from GenBank representing all the human HBV genotypes (>50 subgenotypes) and multi-aligned using the Clustal W algorithm in Bioedit (BioEdit v7.2.6.1)[26]

Neighbour-joining phylogenetic trees were constructed using the MEGA software, version 5.0 [27] and evaluated by bootstrap resampling with 1000 replicates.

RESULTS

Demographics and clinical characteristics

Serum was collected from 84 patients, and genotypic analysis was able to be performed on samples from 76 patients. Clinical data has been presented only for those for whom a genotype was available. Eight patients were excluded; six as they were PCR negative, one had no serum available and one for whom the country of origin was uncertain.

There were 48 males (63%) and 28 females (37%) included in the analysis (Table 1). Date of birth was available for 64/76 (84%), with a median age of 36 years. The median age amongst the largest genotypic group (C1) was also 36. Most were of Karen ethnicity (66/76, 87%), two were of Burmese ethnicity, and for eight patients the ethnicity was unknown. Viral loads were available for 71/76 (93%) of the samples. Amongst those who had transient elastography (n = 24), the median score was 6 kPa (range from 3.3 – 21.3kPa). Cirrhosis was identified in one patient (stiffness 21.3kPa). This patient was on treatment with entecavir and did not have clinical or biochemical markers to suggest cirrhosis.

Disease staging (Table 2) showed 10 patients in the immune tolerance phase, three patients undergoing immune clearance, 26 patients in the immune control phase and 23 patients in the immune escape phase. An additional 15 patients were being treated with antiviral therapy; 10 with entecavir, four with pegylated interferon and one with tenofovir (Table 1 and 2).

Genotyping

Initial genotyping revealed 68 patients with genotype C (89.5%) and eight patients with genotype B (10.5%). Phylogenetic analysis revealed the large majority of the genotype C infections were of subgenotype C1 (67) and one case of C8. Of the genotype B infections, 4 were B7, 3 were B5 and one could not be subgenotyped. Amongst those with genotype C1 disease, the median age of those with HBV eAg positive disease was 29 in the immune tolerance phase and 34 years in the immune clearance phase. The median age of those with

immune HBV eAg negative disease was 41 in the immune control phase and 39 years in the immune escape phase.

Mutational analysis

Sequencing of the HBV polymerase gene (and overlapping surface gene) revealed no mutations associated with antiviral resistance. Fifteen patients were on treatment, ten of these had genotyping done on blood samples taken before treatment was commenced and three in the early months after treatment commencement. One patient treated with entecavir had genotyping done after 18 months of treatment when they had been non adherent for many months and one patient had a previous 12 months course of Pegylated interferon 3 years before the sample used for genotypic analysis. HBV surface gene mutations were detected in 10 patients with subgenotype C1 (Table 3), and these included stop codons at positions sW172 (1 patient)[28], sW182 (3 patients) and sL216 (5 patients), as well as mutations sP120T (1 patient) and sG145R (2 patients) that are known to be associated with HBIg or vaccine escape mutants.[29,30]

HBV BCP/PC sequencing was obtained for 71/76 (93%) patients. BCP and/or PC mutations were identified in 57/71 (80%) of PCR positive patients (Table 3). The most common mutations identified were the PC G1986A with BCP A1762T and/or G1764A (25/57, 44%), the BCP mutations alone (20/57, 35%) and the PC mutation alone (7/57, 12%). A PC start mutation was identified in 2 patients and a 1 nucleotide insertion in PC at amino acid 12 was identified in 3 patients (Table 3).

DISCUSSION

Our results revealed predominantly HBV genotype C in this opportunistic sample of Victorian immigrants from Myanmar. This accounted for 89% of the population, making this

a remarkably homogenous virological population. [12] In particular we identified that the genotype subtype C1 is the predominant sub-genotype in this population. This has also been identified in those with HBV living in Myanmar. [8] HBV isolates from immigrants from Myanmar living in Victoria would likely reflect the HBV from immigrants from Myanmar to Australia as a whole. Genotype C is known to be associated with increased risk of development of HCC [12]. Interestingly the median age of patients with both HBeAg positive and HBeAg negative disease is very similar to studies of Korean patients with genotype C2, showing a mean age of 31 and 40 years respectively. [31] It is therefore interesting that a high proportion of individuals remain HBeAg positive at a relatively advanced age. This suggests that seroconversion may be a relatively late phenomenon in this genotype and population.

Of the samples that were successfully amplified for BCP/PC sequencing, a high proportion (80%) harboured BCP and/or PC mutations. In HBV genotype C, the A1762T/G1764A dual mutations have also been associated with an increased risk of HCC. [12] These results highlight the importance of following HCC screening recommendations in the population of immigrants from Myanmar, who are likely to have genotype C virus. The median transient elastography score was 6.0kPa and only one patient had a transient elastography score suggestive of cirrhosis (21.3). This suggests that the majority of individuals had mild liver disease and is counter to prior reports suggesting genotype C infections being associated with more advanced liver disease although larger studies of liver fibrosis in this genotype are needed to confirm this.

It is notable that 15/76 (18%) of patients were on treatment which would be expected amongst a population with HBV. [32] Of note a further 26 untreated patients were in a stage of disease where HBV treatment would be considered standard of care (three patients in immune clearance and 23 patients in the immune escape phase).[33] It is unclear how long these

individuals had been in these active phases of disease but suggests that at least some individuals may have had unrecognised active disease for prolonged periods.

This has been well recognised as a significant problem within Australia, with epidemiological data suggesting the majority of patients with HBV are not receiving appropriate monitoring and management. [32] It has been recognised that under treatment of patients with HBV is widespread, both in the community and tertiary hospitals [32,34,35] and this finding suggests further efforts are necessary to ensure patients are identified early and receive timely optimal care. This is particularly important given the association of Genotype C virus with HCC and advanced liver disease.

Limitations to the study include that the sample size was small and numbers for non C1 genotypes did not allow for between groups statistical comparisons. In addition data was collected retrospectively, some information was incomplete, and most patients did not have a formal assessment of fibrosis stage. However, these findings are important as they have implications for patient monitoring and treatment. They particularly highlight the need for screening for HCC given the potential for the development of liver cancer in genotype C1 disease. They also illustrate the issue of people with HBV potentially not receiving optimal therapy in a timely fashion.

In summary, patients from Myanmar who migrate to Australia are predominantly infected with genotype C (especially C1) virus and may not be receiving timely therapy. This highlights the need to increase efforts to bring these individuals into the cascade of care. In addition, in order to better guide clinical care and decrease the morbidity and mortality of HBV, improved clinical guidelines need to be informed by further studies into the natural history of various specific genotypes and subtypes of HBV.

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Table 1: Demographic data and pathology results

		All	**B (not subtyped)**	**B5**	**B7**	**C1**	**C8**
Total		76*	1	3	4	67	1
Male sex		48	1	2	2	42	1
Age (years, median)		36	28	41	35	36	40
Pathology results							
(median)	Viral load (IU/mL)	3248	NA	469	451	3768	22823
	ALT (IU/L)	29	24	42	23	29	22
	AST (IU/L)	28	34	34	22	27	32
	Bilirubin (umol/L)	10	9	6	10	10	17
	Albumin (g/L)	42	45	43	43	42	40
Transient elastography	Yes	24	0	0	2	22	0

	Median stiffness (kPa)	6			5	6	
Treatment	Entecavir	10			1	8	1
	Tenofovir	1				1	
	PegINF	4		1		3	
Serology	HBeAg pos	20	0	1	1	17	1
	HBeAg neg	56	1	2	3	50	0
	HBeAb pos	31	0	1	2	28	0
	HBeAb neg	12	0	1	1	9	1
	HBeAb NA	33	1	1	1	30	0
	HBe seroconverted**	31	0	1	2	28	0

* For 6/84 patients no genotype information was available, 1 individual (ISI073) was removed from study as country of origin uncertain, one patient has insufficient serum, these 8 patients were excluded from the analysis

** defined as HBeAg neg and HBeAb pos

Table 2: Stages of disease

	Number (N = 76)	HBeAg pos/neg	HBeAb pos/neg/NA	Viral Load (IU/ml) median (IQR)	ALT (IU/L) median (IQR)
Immune Tolerance	10	10/0	0/5/5	$1.75 *10^8$ ($1.8 – 3.2*10^8$)	32 (16 – 60)
Immune clearance	3	3/0	0/0/3	149 (137 – 10827)	25 (25 -49)
Immune control (low replicative)	25	0/25	14/1/10	532 (222 -703)	30 (21-43)
Immune escape (reactivation)	23	0/23	11/1/11	6717 (3122 - 15092)	24 (19-30)
On treatment	15	7/8	6/5/4		36 (22-65)

NA = Not available, IQR = Interquartile range

Note those with incomplete data have been classified as the closest match. Viral loads are not included for patients who started treatment**.**

Table 3: Frequency of BCP/PC mutations according to sub-genotype

BCP	PC	PC Start	Insertion in PC	B5 (n=3)	B7 (n=4)	B? (n=1)	C1 (n=67)	C8 (n=1)	Total (n=76)
A1762T and/or G1764A					**1**		**18**	**1**	**20**
	G1896A				**1**		**6**		**7**
		M1 L/T					**2**		**2**
			1nt (aa12)				**2**		**2**
A1762T and/or G1764A	**G1896A**				**1**	**1**	**23**		**25**
A1762T and/or G1764A			**1nt (aa12)**				**1**		**1**
Total					**3**	**1**	**52**	**1**	**57**