Mixed-genotypes Infections with Hepatitis C Virus in Hemodialysis Subjects

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SUMMARY

Mixed-genotypes hepatitis C virus (HCV) infections are normally ignored in chronic hemodialysis patients. The aim of this study is to investigate the prevalence of mixed-genotypes infections among hemodialysis patients in Pahang province, Malaysia. Reverse-transcription and polymerase chain reaction methods were performed using two different sets of primers, targeting the 5' untranslated region and non-structural 5B region. Target region base sequences were obtained by direct sequencing. Discrepancy in outcomes from phylogenetic analysis of both regions suggests double infections. Of 40 subjects in eight hemodialysis centres, evidence of mixed-genotypes infections was found in 5 subjects (12.5%) from three different centres. Four patients were infected with mixed genotypes 3 and 1 and one with genotypes 3 and 4. Cases of mixed HCV genotypes infection were considered high among hemodialysis patients in Pahang. However, further investigation is needed to confirm whether they are true mixed infections or perhaps infection with recombinant virus and also to assess the clinicopathologic characteristics of the infection.

KEY WORDS:
Hemodialysis, hepatitis C virus, mixed-genotype infection

INTRODUCTION

Maintenance hemodialysis patients are exposed to the risk of HCV infection with a prevalence that substantially exceeds that in the general population and in peritoneal dialysis patients[1]. The risk of infection has been associated with prolonged vascular access and the potential for exposure to contaminated equipment.

Hepatitis C virus (HCV) is a blood-borne disease virus, classified in the Flaviviridae family. It contains a positive sense RNA genome with one open reading frame (ORF) that codes for 10 polypeptides (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B), flanked by 5' and 3' untranslated regions (UTRs). Based on the viral nucleotide sequence, HCV has been classified into six major genotypes and numerous subtypes [2]. HCV genotyping is a very useful method for determining the source of HCV transmission in infected hemodialysis patients [3, 4]. Direct sequencing and phylogenetic analysis of the partial or complete genome has provided a reliable method for HCV classification. The diversity of the viral genotypes is well documented, where genotypes 1, 2 and 3 are distributed globally, while others are endemic to different geographical areas[5-7]. HCV replicates in an infected person as a complex of different but closely related viral variants, referred to as quasispecies [8]. In some HCV-infected patients, more than one genotype can be found, these are called mixed-genotype infections [9-11].

Monoinfections with a single HCV genotype in hemodialysis patients is commonly described; however, mixed infections with different HCV genotypes is rarely addressed by most genotyping assays, since these are designed to identify only the dominant HCV genotype in the population [12, 13]. Thus, this may have a significant impact on the interpretation of differences in biological and clinical data obtained from various HCV genotype infection [14]. A study of mixed-genotypes HCV infection in chronically infected intravenous drug users, hemodialysis patients and hemophiliacs from Sweden and Russia has suggested that the frequency of these infections is very low, even in high-risk groups [15]. In contrast, a study from Canada concluded that utilising a semiautomated genotyping method is useful in detecting mixed-genotype infections in blood donors[10]. Mixed-genotypes infections have also been commonly reported in parenterally transmitted infection risk groups [13, 16, 17].

In Pahang, no HCV genotyping study on hemodialysis patients was previously performed. Thus, we conducted this study on chronically infected hemodialysis patients in eight hemodialysis centres in Pahang province to determine the prevalence of mixed-genotypes infection cases and correlate the prevalence with the location of the centres, duration of infection in years and patients' demographic factors.

MATERIALS AND METHODS

The study procedure was approved by the Ethics Committee of the Faculty of Medicine, International Islamic University Malaysia. Patients involved in this study were informed about its purpose and a signed informed consent was taken. Forty (40) serum samples were collected from HCV infected hemodialysis patients from 8 different dialysis centers in Pahang. History of blood transfusions and HCV screening test results were obtained from each patient's file record.

RNA extraction was carried out using QIAamp Viral RNA extraction kit according to the manufacturer's instructions (Qiagen, Germany). RT-PCR was performed, targeting the 5'UTR and NS5B regions. The 5'UTR sequence was amplified using HCV-F primers (forward; 5'

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AGTGTTGTGCAGCCTCCAG 3′) and HCV-R primer (reverse; 5′ ACTGCCTGATAGGGTGCTTG 3′), generating a 212 bp amplicon. Samples with negative outcomes in the RT-PCR were tested in a second round of semi-nested RT-PCR using HCV-nF primers (forward; 5′ CGGTGAGTACACCGGAATTG 3′) which gave a 153 bp amplicon. Meanwhile, a 400 bp segment of the NS5B sequence was amplified using primers universal to genotypes 1 to 5, adopted from a previous study[3]. Another pair of NS5B primers, 7946F (forward; 5′ TTAACCACATCAACTCCGTG 3′) and 8643R (reverse; 5′ CCGAATACCTGGTCATAGC 3′), was used specifically to amplify HCV genotype 6 sequence, generating a 695 bp amplicon in the RT-PCR. cDNA was synthesized using ImpromII™ reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's protocol. PCR was carried out under standard conditions, using 2.5U of Go Taq Polymerase (Promega, Madison, WI, USA), 1x buffer, 0.75 mM of $MgCl_2$, 0.2 mM of dNTP and 0.4 ρmol of each primer in a total volume of 50 µl. Five µl of cDNA was added into the PCR mixture. Thermal profile for the amplification of both regions was as follows: initial denaturation at 94ºC for 3 minutes; 35 cycles with denaturation at 94ºC for 1 minute, annealing at 56ºC for 1 minute and elongation at 72ºC for 3 minutes. The reaction was terminated with a final extension at 72ºC for 5 minutes.

The amplicons were purified with the MinElute gel extraction kit (Qiagen, Valencia, CA, USA). The purified products obtained from PCR amplification were sequenced using the same primers as PCR. Each amplicon was sequenced twice from both directions on an ABI Prism 3100 automated DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequences alignment and phylogenies were analyzed using MEGA 4.1 software package (www.megasoftware.net). The sequencing results were aligned with ClustalW Alignment together with known sequences obtained from NCBI homepage. Pairwise evolutionary distance matrices for the 5′UTR and NS5B nucleotide sequences were computed using the Jukes-Cantor algorithm and the neighbour-joining method for tree drawing. The reliability of phylogenetic classification was evaluated by a 1,000-replication bootstrap test.

Data were analyzed using SPSS version 17.0. The 95% confidence interval (95%CI) of prevalence was estimated using confidence interval calculator available online (http:wwwdimensionresearch.com). Fisher's exact test with two-sided P values was used to compare between frequencies of variables. $P<0.05$ was considered as significant.

RESULTS

Subjects studied

Of the 40 patients, 24 (60.0%) were men and 16 (40.0%) were women. Duration of HCV infection ranged from four months to 17 years (Mean = 6.3 years; SD = 3.7 years). The median and mode of classified age groups ranged from 46 to 55 years (n=16). The probable sources of infection include multiple blood transfusion (75.0%), renal transplantation (5.0%), nosocomial transmission (5.0%) and unknown (15.0%). Diabetic patients were recognized in 6 of them (15.0%) and 8 (20.0%) of hepatitis B coinfection were identified. No HIV coinfected patients were present. Three of these patients were positive for anti-HCV before their first dialysis. All HCV infected subjects were previously screened for HCV infetion with a commercial third-generation ELISA (MONOLISA® Anti-HCV PLUS Version 2, BIO-RAD) and in this study the serological tests were confirmed by the RT-PCR assay. Prevalence of HCV infection varied between centers ranging from 2.7% to 21.5% with an overall prevalence of 11.6% in the eight centers (Table I).

HCV genotyping and mixed-genotypes infection cases in hemodialysis centers

The 5′UTR and NS5B sequences were both obtained from 33/40 hemodialysis patients, 5′UTR only for 3 and NS5B for the other 3. Collectively, sequencing of either or both regions was successful for 39 out of 40 patients. In the group of 33 patients, 28 had single HCV genotype infection with the following frequencies: genotype 3 in 19 (67.9%), genotype 1 in 7 (25.0%), genotype 4 in 1 (3.6%) and genotype 6 in 1(3.6%) (Table I, Fig 1). Cases of neither genotype 2 nor 5 were found in the study panel. The NS5B sequence data of all isolates in this study were submitted to GenBank. The accession numbers of the sequences are JN207131 to JN207162, JN038188 to JN038189 and HQ450380 for HCV genotypes 3 & 1, 4 and 6, respectively.

Of 8 centers, mixed infection cases were detected in 3 of them. All patients seroconverted during their maintenance dialysis. Mixed infection with genotype 3 & 1 was detectected in 4 patients and genotypes 3 & 4 in 1 patient. Comorbidity with diabetes mellitus and HBV coinfection was not statistically significant in mixed infection cases (Table II).

DISCUSSION

The present study was performed on 40 HCV seropositive samples from chronic hemodialysis patients in Pahang province. Phylogenetic inferences from 5′UTR and NS5B sequences of 28 samples revealed the predominance of HCV genotype 3 followed by HCV genotype 1 (Fig 1). HCV genotype 3 has been associated with intravenous drug users in Western Europe [18]. Conversely, genotype 3 infection is predominant in blood donors in Thailand [19] and genotype 1 in chronic HCV carrier from Singapore [20]. Very limited data on HCV genotypes prevalence in hemodialysis subjects is available from South East Asian countries. The non-existence of genotypes 2 and 5 in the present study suggested that they are not yet introduced to the studied hemodialysis population in Pahang.

Five of our cases had mixed-genotypes infections (12.5%), of which four were infected with genotypes 3 and 1 and one case with genotypes 3 and 4. HCV genotype 4, very frequent in Middle East countries [21], was found in only two patients, including the one with mixed infection. However, confirmation of these findings requires a larger study with more subjects. No significant differences were observed regarding associated risk factors between single and mixed-genotypes cases probably suggesting that all chronic hemodialysis patients have an equal chance of developing either single or mixed infection (Table II). Similar findings were reported in a study by Giannini *et al.*, in 1999 of mixed-

Table I: Prevalence single and mixed-genotypes HCV infections in hemodialysis centers in Pahang province

Hemodialysis Centers	Prevalence of HCV infection (%) (n= 40)	Mixed-genotypes infection, 5'UTR + NS5B, (n = 5)	Mono infection, 5'UTR + NS5B (n = 28)			
			*G1 (n = 7)	G3 (n = 19)	G4 (n = 1)	G6 (n = 1)
Tengku Ampuan Afzan Hospital (HTAA)	14/65 (21.5)	G3+G1 (2) & G3+G4 (1)	3	6	-	-
Sultan Haji Ahmad Shah Hospital (SHASH)	9/53 (16.9)	-	-	6	-	-
Raub Hospital	1/37 (2.7)	-	1	-	-	-
Pekan Hospital	3/32 (9.4)	G3+G1(1)	-	1	-	-
Jerantut Hospital	4/31 (12.9)	G3+G1(1)	2	1	-	-
Kuantan Specialist Hospital (KSH)	2/27 (7.4)	-	-	1	1	-
Pusat Hemodialysis Islam Makmur (PHIM)	4/60 (6.7)	-	-	3	-	-
Pahang Buddist Association (PBA) Hemodialysis	3/41 (7.3)	-	1	1	-	1

*G = Genotype

Table II: Risk factors associated with single and mixed HCV genotypes infections

Parameters	Total (n = 33) (%)	Mixed-genotypes infection (n = 5)(%)	Monoinfection (n = 28) (%)	P value
Age				
>45	26(78.8)	3 (60.0)	23(82.1)	P = 0.28
<45	7(21.2)	2 (40.0)	5(17.9)	
Gender				
Men	21(63.6)	4 (80.0)	17(60.7)	P = 0.63
Women	12(34.4)	1 (20.0)	11(39.3)	
Duration of infection (until 2009)				
>6 years	16(48.5)	3 (60.0)	13(46.4)	P = 0.66
<6 years	17(51.5)	2 (40.0)	15(53.6)	
Multiple blood transfusions				
Yes	32(97.0)	5(100.0)	27(96.4)	P = 1.00
No	1 (3.0)	0 (0)	1 (3.6)	
HBV coinfection				
Yes	5(15.2)	0 (0)	5(17.9)	P = 0.56
No	28(84.8)	5 (100)	23(82.1)	
Diabetes mellitus				
Yes	5(15.2)	1 (20.0)	4(14.3)	P = 1.00
No	28(84.8)	4 (80.0)	24(85.7)	

genotypes HCV infection in patients with chronic liver disease, which indicated the occurance of mixed-genotypes infections in the absence of discernible risk factors [22].

Additionally, the occurrences of mixed infection were not associated with the level of HCV prevalence nor with the geographical location of each dialysis centre (Table I). Hemodialysis patients commonly become anaemic, thus multiple blood transfusions may lead to reinfection with different HCV genotypes or variant of a similar genotype. Mixed-genotypes infections have been frequently reported in patients with chronic hepatitis C especially in those with parenterally transmitted infections through multiple blood transfusions[10, 13, 16, 17], suggesting a nosocomial mode of transmission. Therefore, strict adherence to standard infection control procedure should be considered in order to reduce the number of seroconversions either whether due to mixed or single infections.

In this study, direct sequencing method was used to detect the mixed-genotypes infection. DNA sequencing has become the gold standard in HCV genotypes investigations.In clinical practice many mixed or recombinant infections are overlooked due to non-availability of reliable commercial genotyping test for their diagnosis. The findings in the present study showed that mixed-genotypes infections within the same individual is of common occurance in the chronic hemodialysis patients. A similar study by Qian et al., (2000) has shown that the prevalence of mixed-genotypes infection in hemodialysis patients is high and can be reliably detected with molecular methods [23].

The treatment of choice for chronic HCV hepatitis is conventional or pegylated interferon, alone or in combination with ribavirin. Predictive outcome of the treatment also relies on the viral genotype. Yet, antiviral therapy in hemodialysis patients remains controversial as no comparative studies have been done to support the decision of an appropriate dosage of treatment[24-30]. Clinical trial is difficult due to the adverse event of severe anaemia caused by secondary haemolysis[24]. Therefore, the local policy of monitoring hepatitis C in hemodialysis patients is yearly ultrasound and alpha-fetoprotein measurement to screen for hepatoma. Patients who are top three in renal transplant waiting list will be referred to a gastroenterologist for interferon treatment; consequently the viral genotype will be determined. However, none of the subjects studied was planned for a renal transplant. Thus, patients in this study were all treatment naïve during the study period. In a previous study, Hadzri *et al.*, (2009) have reported the most common HCV genotypes found in HCV infected patients in Hospital Tengku Ampuan Afzan, Kuantan, Pahang and Hospital Sultanah Nur Zahirah, Kuala Terengganu, Terengganu, which are genotypes 3 followed by 1[31]. However,

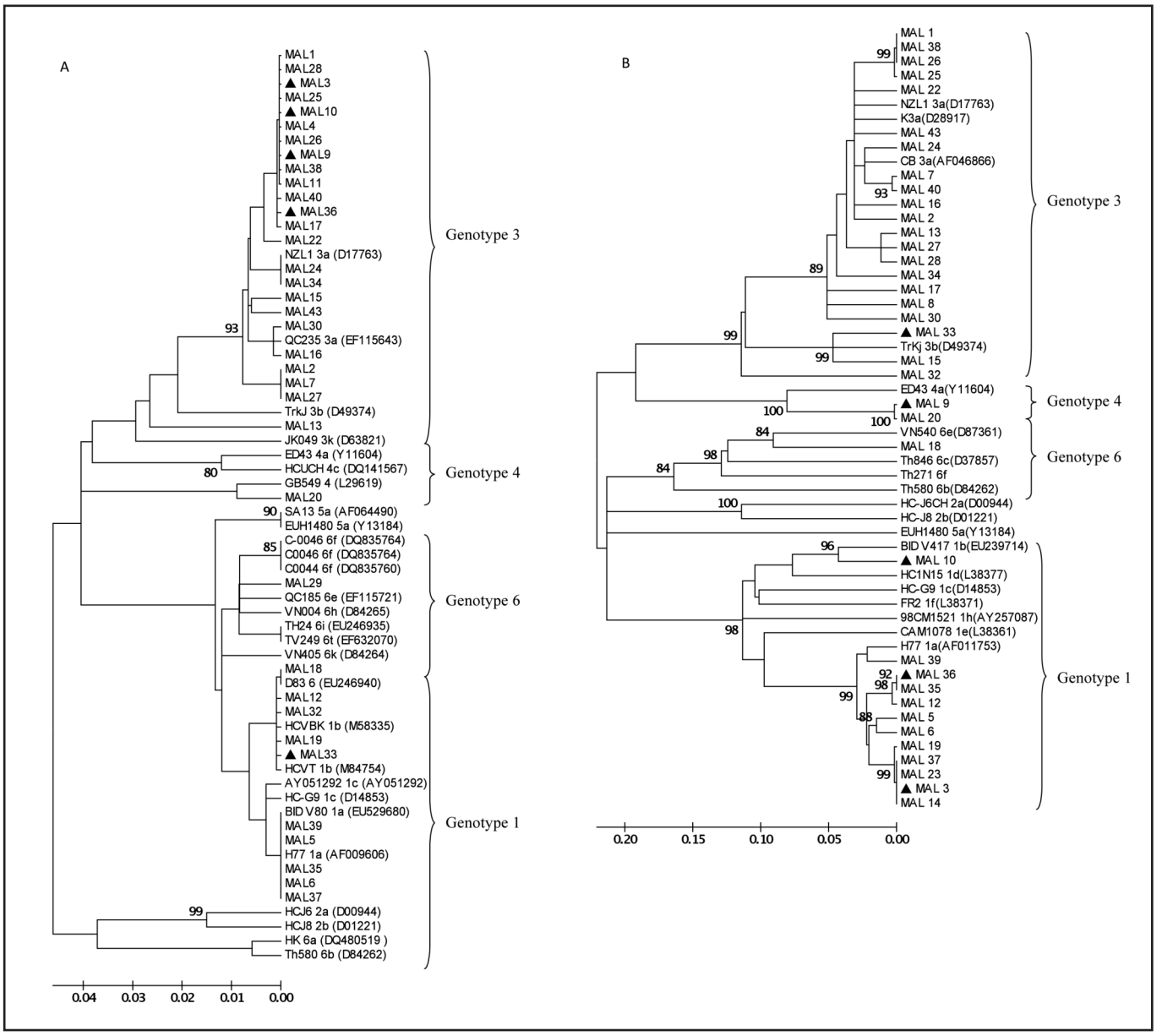

Fig. 1: Phylogenetic classification of HCV isolates from hemodialysis patients in Pahang province. The phylogenetic trees were constructed using Jukes-Cantor model with neighbour-joining method. Bootstrapping values, higher than 80, are shown next to the branch. Isolates of the reference sequences are identified by their name of isolates and Genbank accession number. Isolates in the study panel are shown with prefix MAL followed by an Arabic numeral. Isolates with discordant classification in both trees are indicated with dark triangle. A. Genotypes classification based on 5'UTR sequences. B. Genotypes classification based on NS5B sequences.

the local prevalence of hepatitis C genotypes specifically in hemodialysis patients has never been reported before.

In this study, the true nature of the mixed-genotypes apparently is yet to be clarified as monoinfection with recombinant HCV variants or true mixed-genotypes infections. The determination of the true classification can be done by obtaining the nucleotide sequences from either multiple genomic region or also the entire HCV genome. Recombinant intragenotypic and intergenotypic HCV also has been described in various high risk groups elsewhere [32-35]. Mixed infection with two or more HCV genotypes in an individual is of great clinical importance as it may result in more severe disease progression. Appreciation of the role of innate [36, 37] and adaptive immune responses [38, 39] in HCV infection is just beginning to emerge. Thus, the existence of mixed-genotypes infection and recombinant variants complicate the understanding of HCV persistence and immunopathogenesis. Mixed-genotypes or recombinant HCV infection was thought to be favourable to escaping from immune surveillance, leading to chronicity. Mutations within hypervariable region 1 (HVR1) have been related to anti-HCV neutralizing antibody responses during primary infection[24, 40]. Recently, HCV genotype 3 has been discovered

to have two novel HVRs, additional to the well-known HVR1, that favour the virus infectivity under positive selecting forces during primary immune response [41]. These findings and others too might explain the involvement of HCV genotypes 3 in the mixed-genotypes infections [9, 16, 42].

CONCLUSION

In conclusion, a considerably strong presence of mixed-genotypes infections is noted in the hemodialysis setting in Pahang. Therefore, the preliminary results obtained in this study are beneficial for future studies on diagnostic and prognostic implications of mixed-genotypes infections in this region, especially those associated with HCV genotype 3 in Pahang, Malaysia.

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