PREVALENCE OF GB VIRUS C / HEPATITIS G VIRUS INFECTION IN PEDIATRIC PATIENTS RECEIVING MULTIPLE TRANSFUSIONS IN SOUTHERN TURKEY*

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SUMMARY: Koçabaş E, Antmen B, Yarkın F, Serin M, Aksungur P, Tanyeli A, Kılınç Y, Aksaray N. (Departments of Pediatric Infectious Diseases, Pediatric Hematology-Oncology, and Microbiology, Çukurova University Faculty of medicine, Adana, Turkey). Prevalence of GB virus C/hepatitis G virus infection in pediatric patients receiving multiple transfusions in Southern Turkey. Turk J Pediatr 1999; 41: 81-90.

The aim of this study was to determine the prevalence of GB virus C (GBV-C) infection in pediatric patients receiving multiple blood transfusions in Turkey where HBV and HCV infections are common. Sera of a total of 148 children, of whom 85 had cancer and 63 hemoglobinopathies, were tested for GBV-C RNA and HCV RNA by RT-PCR and for antibodies to HBV and HCV. Demographic and clinical information as well as laboratory results were recorded for the patients (81 boys, 67 girls, aged 1-19 years). HBsAg positivity was found in 23 (15.5%) patients, HBV DNA positivity in 12 (8.1%), HCV RNA positivity in 9 (6.7%), and GBV-C RNA positivity in 4 (2.7%). There was no significant difference in the GBV-C RNA positivity between patients with cancer (3.2%) and patients with hemoglobinopathies (2.4%) ($p > 0.05$). GBV-C RNA was found in 4 (3.1%) out of 127 patients who had received transfusions, but it was not found in any of 21 patients who had not received transfusions. However, there was no relationship between GBV-C RNA positivity and the number of transfusions. Two of the patients with GBV-C RNA had high levels of ALT (ALT > 40 IU). In these two patients, neither HBV DNA nor HCV RNA were detected by PCR, and serological tests were also negative for these agents. We concluded that pediatric patients who had multiple transfusions in Turkey are at risk of being infected with GBV-C, in addition to HBV and HCV. Investigation of GBV-C RNA in patients with high ALT levels in the absence of other viral markers may be useful. *Key words: GBV-C, pediatric cancer, hemoglobinopathy.*

Screening of blood donors for hepatitis C virus (HCV) using sensitive serological methods has greatly reduced the incidence of non-A, non-B hepatitis that occurs after transfusions. On the other hand, the fact that post-transfusional hepatitis not related to viral hepatitis A-E is still seen in two to three percent of cases has led to studies concentrating on other hepatitis viruses[1,2].

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In 1967, a viral GB agent which could cause hepatitis was reproduced in tamarins (small primates)[3]. In 1995, the presence of two genomes resembling Flaviviruses was detected in the serum of tamarins infected with the GB agent using a new PCR method called representational difference analysis (RDA). These two agents were called GB virus A (GBV-A) and GB virus B (GBV-B). During the same year, a new virus, GB virus C (GBV-C), which showed a 59 percent nucleic acid and a 64 percent amino acid homology with GBV-A, was isolated from the serum of a patient with non A-E hepatitis using reverse transcriptase polymerase chain reaction (RT-PCR). Other studies have reported that GBV-C has a 29 percent amino acid homology with HCV and a 28 percent amino acid homology with GBV-B and that this is the first in a series of viruses which have so far escaped the notice of researchers[4-7]. In 1996, a new RNA viral genome, 9393-nucleotide in length, was found in the serum of a patient with chronic non-A, non-B hepatitis using the RT-PCR; this virus was named hepatitis G virus (HGV)[5-8]. Because of a 90 and 95 percent similarity in nucleotide and amino acid levels of the HGV and GBV-C prototypes, respectively, it has been suggested that these are two different isolates of the same virus. While GBV-A and GBV-B are viruses of the tamarin, it has been reported that GBV-C is a human virus belonging to the Flaviviridae family due to its genomic organization[4-9].

HGV/GBV-C infection is frequently seen in patients with hemophilia and thalassemia who have received multiple transfusions of blood and blood products, in those who use intravenous drugs, and in patients with acute and chronic non A-E hepatitis resulting from blood transfusions[8, 10]. The transmission of HGV/GBV-C occurs parenterally (in those receiving blood and blood products, drug addicts and those undergoing hemodialysis) and vertically from mother to infant[11, 12]. At this time, there are no serological tests which can detect carriers of HGV/GBV-C. Currently, the only reliable method for detection of viral RNA in serum or other infected fluids or tissues is specific reverse transcriptase polymerase chain reaction (RT-PCR).

Previously, we have shown that children with hemophilia and cancer are at higher risk for hepatitis B virus (HBV) and hepatitis C virus (HCV) infection in Turkey, where HBV and HCV infections are common in the general population and screening of blood procedures for these agents is not effective[13, 14]. Because GBV-C is transmitted by transfusion as HBV and HCV are, it can be expected that pediatric patients receiving multiple transfusions in Turkey are at a higher risk for GBV-C infection too. Our aim in this study was to investigate the prevalence of GBV-C infection in Turkish pediatric patients with hemoglobinopathy and cancer who had received multiple blood transfusions.

Material and Methods

This study was carried out between October and December 1996 in the Pediatric Hematology-Oncology Department of Çukurova University Faculty of Medicine

located in southern Turkey. A total of 148 children, of whom 85 had cancer (53 with acute leukemia; 12 lymphoma; and 20 solidtumors) and 63 had hemoglobinopathies (46 with thalassemia and 17 sickle cell anemia), were investigated. All the patients with cancer were under maintenance therapy. Demographic (age and sex) and clinical information (time elapsing since diagnosis, number of transfusions, and history of operation, jaundice, and HBV vaccination) as well as laboratory results were recorded for the patients (81 boys, 67 girls, aged between 1-19 years). Serum samples which were taken for the detection of serum alanine aminotransferase (ALT) and hepatitis markers and for PCR studies were kept at -20 °C until testing was done. The ELISA method was used for the detection of HBsAg, anti-HBs and anti-HBc (Murex, England) and the III generation ELISA method for the detection of anti-HCV antibodies (UBI, New York, USA).

Detection of GBV-C RNA with Nested RT-PCR

Extraction of RNA

The extraction of the RNA was carried out as follows: 100 µl of the serum of each patient was mixed with 400 µl lysis buffer containing 4M guanidine thiocyanate, 25 mM Na citrate, 0.5 percent sarcosyl, and 3 µl DDT. The mixture was incubated at 60 °C for 10 minutes. After 500 µl of isopropyl alcohol was added to each tube, they were centrifuged at 13,000 rpm for 15 minutes. After removal of the upper phase, 1 µl of 70 percent ethanol was added to the pellet and the tubes were placed in dry ice for 10 minutes. The tubes were than again centrifuged at 13,000 rpm for 10 minutes and the upper phase was discarded. The precipitated RNA was suspended in 500 µl of 1xPCR buffer without gelatin.

Reverse transcriptase (RT) and Amplification of cDNA

In order to produce the complementary DNA (cDNA) of the extracted RNA, 2 µl of the RNA extract was mixed with the lower master mix (0.5 µl RNase block, 0.5 µl containing 10 U reverse transcriptase, 4 µl dNTP, 1 µl primer G9, 2 µl of 25 mM $MgCl_2$, and 1 µl RT buffer). This reaction mixture was incubated for one hour at 43 °C in a M.J. Research (PTC-150) type thermal cycler. Later, in order to inactivate the enzymes, the mixture was kept at 99 °C for five minutes and then cooled in dry ice. At the end of this reaction, the upper master mix containing 32.5 µl sterile distilled water, 1 µl 10xPCR buffer, 3 µl $MgCl_2$ (25 mM), 0.5 µl Taq polymerase (2.5 U), 1 µl primer G8 and 1 µl primer G9 was added to the lower master mix. The first round of PCR was performed for 35 cycles (94 °C, 30 sec denaturation; 55 °C, 30 sec annealing; 72 °C, 60 sec elongation) using 50 µl of this reaction mixture. The final elongation was carried out at 72 °C for eight minutes.

The second round of PCR for HGV was carried out with 5 µl of the amplification product, 5 µl 10xPCR buffer, 3 µl (25 mM) $MgCl_2$, 0.5 µl dNTP, 0.5 µl Taq

polymerase, 1 µl primer G10, 1 µl primer G11 and 34 µl sterile distilled water. Poymerase chain reaction (PCR) was performed for 30 cycles with each cycle consisting of the same schedule as the first-round PCR, except for the primer extension at 72 °C which was carried out for 45 sec. The final elongation was then carried out at 72 °C for eight minutes[15].

Analysis of the Amplification Products

The amplification products were analyzed using electrophoresis (6 volts/hour) in two percent agarose gel. All of the amplification products were separated throughout the agarose and stained with etidium bromide. The bands were seen on UV-transilluminator (Vilber Lourmat). A ϕx174/HeaII DNA marker was used for the molecular weight measurements of the viral DNA. Expected lengths for products of the first and second rounds of the PCR were 158 bp and 83 bp, respectively. The samples in which the 83 bp DNA fragments were detected were accepted as being positive for GBV-C RNA. A positive patient serum control and distilled water for a negative control were included in the cDNA transcription and the PCR procedures.

The primers used for the alpha helicase region of GBV-C are as follows:

Primer G9: 5'-TCYTTGATGATDGAACTGTC-3'
Primer G8: 5'-TATGGGCATGGHATHCCYC-3'
Primer G10: sense, 5'-CATTCVAAGGCGGAGTGYGA-3'
Primer G11: antisense, 5'-TCYTTACCCCTRTAATAGGC-3'

(Y, mixture of T and C; D, mixture of A, G and T; H, mixture of A, C, and T; V, mixture of A, C and G; R, mixture of A and G).

The amplification of HBV DNA in serum was carried out using PCR with the primers for the core region of the HBV genome which are HBV CA1:GCT TTG GGG CAT GGA CAT TGA CCC (1893-1916) and HBV CA2B. Biotin-TGA TAA GAT AGG GGC ATT TGG TGG (2302-2325). The amplification products were made visible using microwells coated with streptoavidin and RNA probes conjugated with biotin (Digene Sharp Signal System, Cat. no. 4601-110).

The HCV RNA in serum was amplified with primers aimed at the conservative 5'-untranslated region of the viral genome using RT-PCR according to the method of Okamato for HCV[16].

The data analyses were carried out using the SPSS packet statistical program including chi-square, Student's t test, Fisher's exact test and nonparametric Mann-Whitney U test. Values $p < 0.05$ were considered to be significant.

Results

A total of 148 pediatric patients including 85 with pediatric cancer and 63 with hemoglobinopathies were investigated in this study. The clinical and laboratory

findings of the patients are shown in Table I. HBsAg positivity was found in 23 (15.5%) patients, HBV DNA positivity in 12 (8.1%), anti-HCV positivity in 17 (11.5%), HCV-RNA positivity in 9 (6.7%), and GBV-C RNA positivity in 4 (2.7%). The mean age and the mean number of transfusions and the prevalence of HBV vaccination in the hemoglobinopathy group were higher than in the cancer group ($p < 0.01$). However, the prevalence of a history of surgery was higher in the cancer group as compared to the hemoglobinopathy group ($p < 0.01$). In contrast to this, there was no significant difference in the positivity of HBV DNA, HCV RNA and GBV RNA between the two groups ($p > 0.05$).

Table I: Clinical and Laboratory Findings in Patients with Hemoglobinopathies and Cancer

	Patients			
Clinical and Laboratory Characteristics	Total Patients (n=148)	Patients with Hemoglobinopathy (n=63)	Patients with Cancer (n=85)	(p)
Mean age (± SD) (years)	8.6 ± 4.3	9.8 ± 4.7	7.8 ± 3.8	<0.01
Male/Female ratio	81/67	34/29	47/38	NS
Mean duration of diagnosis (± SD) (months)	49.2 ± 51.5	48.7 ± 51.1	49.7 ± 52.2	NS
Mean number of transfusion (± SD)	33.5 ± 59.9	72.1 ± 76.6	4.9 ± 4.7	<0.001
Mean level of ALT (± SD) (IU)	49.9 ± 72.5	42.4 ± 51.4	55.4 ± 84.8	NS
ALT > 40 IU (+) (no, %)	50 (33.8)	18 (28.6)	32 (37.6)	NS
History of surgery (+) (no, %)	30 (20.3)	6 (9.5)	24 (28.2)	<0.01
History of jaundice (+) (no, %)	2 (1.4)	0 (0)	2 (2.4)	NS
HBV vaccination (+) (no, %)	5 (3.4)	5 (7.9)	0 (0)	<0.01
HBsAg (+) (no, %)	23 (15.5)	8 (12.7)	15 (17.6)	NS
Anti-HBs (+) (no, %)	34 (23.0)	14 (22.2)	20 (23.5)	NS
Anti-HBc (+) (no, %)	15 (10.1)	8 (12.7)	7 (8.2)	NS
HBV DNA (+) (no, %)	12 (8.1)	5 (7.9)	7 (8.2)	NS
Anti-HCV (+) (no, %)	17 (11.5)	8 (12.7)	9 (10.6)	NS
HCV RNA (+) (no, %)	9 (6.1)	4 (6.3)	5. (5.9)	NS
GBV-C RNA (+) (no, %)	4 (2.7)	2 (3.2)	2 (2.4)	NS

The clinical and laboratory findings of the patients according to the number of transfusions received are shown in Table II. The mean duration of time which elapsed after diagnosis was longer in the group who had received transfusions (52.9 ± 53.9 months) in comparison to those who had not received transfusions (26.7 ± 24.3 months) ($p < 0.05$) (Table II). However, the frequency of a history of surgery was higher ($p < 0.05$) in patients who had not received transfusions (52.4%) in comparison to those who had received transfusions (15%). GBV-C RNA was found in four (3.1%) out of 127 patients who had received transfusions, but it was not found in any of the 21 patients who had not received transfusions.

The clinical and laboratory findings in GBV-C RNA positive patients are shown in Table III. Two of these patients had thalassemia; 1, acute lymphoblastic leukemia (ALL); and 1, non-Hodgkin's lymphoma (NHL). The time after diagnosis

for these patients ranged from seven months to 16 years. None of the patients had HBV or HCV markers. Two patients with GBV-C RNA seropositivity had high ALT levels (ALT > 40 IU).

Table II: Clinical and Laboratory Findings of Patients According to the Number of Transfusion

Clinical and Laboratory Characteristics	Those with no Transfusions (n=21)	Patients with 1-10 Transfusions (n=76)	Patients with 11 or more Transfusions (n=51)	(p)
Mean age (± SD) (years)	8.7 ± 3.5	2.4 ± 3.6	10.7 ± 4.7	p<0.001
Male/Female ratio	6/15	39/37	36/15	p<0.05
Mean duration of diagnosis (± SD) (months)	26.7 ± 24.3	44.7 ± 45.8	65.1 ± 62.6	p<0.05
Mean number of transfusion (± SD)	0	5.0 ± 2.75	89.8 ± 75.0	p<0.05
Mean level of ALT (± SD) (IU)	47.1 ± 59.7	49.5 ± 85.4	51.4 ± 55.5	NS
ALT > 40 IU (+) (no, %)	7 (33.3)	24 (31.6)	19 (37.3)	NS
History of surgery (+) (no, %)	11 (52.4)	13 (17.1)	6 (11.8)	p<0.05
History of jaundice (+) (no, %)	0	1 (1.3)	1 (2.0)	NS
Hepatitis B vaccination	0	0	5 (9.8)	NS
HBsAg (+) (no, %)	3 (14.3)	15 (19.7)	5 (9.8)	NS
Anti-HBs (+) (no, %)	1 (4.8)	21 (27.5)	12 (23.5)	NS
Anti-HBc (+) (no, %)	1 (4.8)	8 (10.5)	6 (11.8)	NS
HBV DNA (+) (no, %)	2 (9.5)	5 (6.6)	5 (9.8)	NS
Anti-HCV (+) (no, %)	2 (9.5)	8 (10.5)	7 (13.7)	NS
HCV RNA (+) (no, %)	1 (4.8)	5 (6.6)	3 (5.9)	NS
GBV-C RNA (+) (no, %)	0	2 (2.6)	2 (3.9)	NS

Table III: Clinical and Laboratory Findings of GBV-C RNA (+) Patients

Clinical and Laboratory Characterics	Patient I	Patient II	Patient III	Patient IV
Sex/Age (year)	F/17	M/3	M/5	M/16
Diagnosis of illness	Thalassemia	ALL	NHL	Thalassemia
Duration of diagnosis	16 years	2 years	7 months	15 years
Number of transfusions	36	1	9	260
ALT level (IU)	81	21	58	21
History of surgery	(-)	(-)	(-)	(+)
History of jaundice	(-)	(-)	(-)	(-)
Hepatitis B vaccination	(+)	(-)	(-)	(-)
HBsAg	(-)	(-)	(-)	(-)
Anti HBs	(+)	(-)	(-)	(+)
Anti HBc	(-)	(-)	(-)	(-)
Anti HCV	(-)	(-)	(-)	(-)
HCV RNA	(-)	(-)	(-)	(-)
GBV-C RNA	(+)	(+)	(+)	(+)
Elapsed time since last transfusion (months)	12	24	6	1

Discussion

Hepatitis G virus is clearly a transmissible agent that may be spread in the same manner as other blood-borne viral agents such as the hepatitis viruses and retroviruses[17]. Studies in the Centers for Disease Control and Prevention have found that among patients in the United States with newly diagnosed non-A, non-B hepatitis, approximately 18 percent were positive for HGV RNA. Most of these patients (approximately 80%) were also infected with HCV[6]. The seroprevalence of HGV is found in 18 percent of patients with hemophilia and thalassemia who are frequently given blood transfusions, as well as in 33 percent of those using intravenous drugs and in 3.1-20 percent of patients undergoing renal dialysis[5, 8, 10].

Limited data regarding the epidemiology of HGV is available. It has been reported that the seroprevalence of HGV/GBV-C in West Africa is 25.9 percent; in Japan, four percent and in Taiwan, one percent[5, 18-20]. A study carried out on voluntary blood donors in the United States reported that the prevalence of GBV-C in donors with normal levels of ALT was 1.7 percent and that the prevalence in those with levels of ALT over 45 IU was 1.5 percent. In various countries, the prevalence of HGV/GBV-C in voluntary blood donors ranges from one to two percent[8].

In studies carried out in Turkey, the rate of GBV-C RNA positivity in adult patients given frequent blood transfusions is reported as 15 percent[21]; in patients with chronic liver disease, 4.6-20.2 percent[22, 23]; and in patients undergoing hemodialysis, 21-36.4 percent[24, 25]. The prevalence of GBV-C in the 148 pediatric cancer and hemoglobinopathy patients investigated in this study was 2.7 percent. There was no significant difference in the prevalence of GBV-C between the patients with cancer (3.2%) and those with hemoglobinopathy (2.4%).

HGV/GBV-C may be associated with acute hepatitis. Chronic hepatitis characterized by elevated serum ALT levels may occur, but chronic infection with no evidence of hepatitis is also common. Hepatitis due to GBV-C infection is often milder than that seen with HCV infection alone. Seventy-five to 80 percent of those who have been infected with GBV-C as a result of transfusions have normal levels of ALT. These with viremia who have normal levels of ALT are thought to be normal carriers. A high level of ALT is usually due to a co-infection with HCV, and the ALT activity is related more to the level of HCV RNA than to HGV/GBV-C RNA[5-7, 17]. While 73 percent of patients with only a HBV/GBV-C infection have no evidence of hepatocellular damage, about 16 percent of them show slight increases in ALT levels[7, 17, 20, 26]. For these reasons, it has been suggested that HGV/GBV-C may not be a primary hepatotrophic agent and that the presence of HGV/GBV-C RNA in the lymphocytes of patients with viremia may lead to the production of hepatitis only under certain circumstances[5, 27]. In this study, it was found that in two (50%) out of four GBV-C RNA positive patients, the level of the serum ALT was 1.5-2 times higher than normal. Even

though a high level of serum ALT is usually due to a co-infection by HCV and HBV[11], in these patients neither HBV DNA nor HCV RNA was detected with PCR, and serological tests were also negative for these agents. Just as chronic hepatitis could be the reason for the higher level of ALT found in these two patients, it may also have been due to the drugs given for non-Hodgkin's lymphoma (NHL) and/or other reasons.

HGV/GBV-C infection is characterized by the presence of persistent viremia and it has been reported in the literature that this may continue for as long as 17 years[5, 17]. In a study of patients who had received a massive blood transfusion because of liver transplantation, GBV-C RNA was detected six to 14 days after the transfusion. The period of viremia lasted for five months to four years in these patients. The most important source for transmission of GBV-C are transfusions of plasma, blood, and erythrocyte and thrombocyte concentrates[28]. In our study, the length of time after diagnosis in the four GBV-C RNA positive patients ranged from seven months to 16 years, and the time that passed between their last transfusion and the time this research was carried out ranged between one months to 2 two years. The GBV-C RNA positivity detected in this study may have been due either to a persistent viremia or to a passive transfer of the agent from a donor with viremia. The presence of a significant length of time since diagnosis in the GBV-C RNA positive patients supports the view that GBV-C may be a cause of a persistent chronic infection.

The facts that patients who were negative for HGV/GBV-C RNA before transfusion developed HGV/GBV-C RNA positivity within such a short a time as two weeks, that in patients who had not received transfusions the incidence of HGV/GBV-C was significantly low, and that after transfusion 10 percent of the patients were infected with HGV/GBV-C clearly indicate that HGV/GBV-C is transmitted by transfusion[6, 7, 26]. This view is supported by the fact that in our study GBV-C RNA positivity was detected in four (3.1%) out of 127 pediatric patients with cancer and hemoglobinopathy who had transfusions, where as all of those who had not had transfusions were negative. The presence of GBV-C RNA was detected in two (4.3%) of 46 thalassemia patients, in one (2.1%) of 48 ALL patients and in one (16.6%) of six NHL patients. In previous studies, no relationship had been found between the number of transfusions and GBV-C viremia[26]. In the present study, no statistically significant relationship was found to exist between viremia and the number of transfusions.

Even though HGV/GBV-C usually causes chronic persistent infections, it may also be eliminated by the immune system within three years from one-third of patients with a normal immune system. On the other hand, in patients with immunosuppression, this period may be longer and persistent infections may be more common[26, 29]. In one study, in which a seven year follow-up was made of seven pediatric patients, three of whom had thalassemia and four sickle cell

anemia it was found that there was a persistent viremia but no sign of liver disease. This situation may be explained by a persistent viremia in patients with a normal immune system and thereby a chronic carrier state[30]. The GBV-C RNA positivity found in the patients in our study may be due to immunosuppression in the cancer group or to multiple blood transfusions in all the patients.

Turkey, which is a developing country, is regarded as intermediate in endemicity for HBV infection. Prevalence of HBsAg reactivity among the general population ranges between 3.9-12.5 percent in different regions of Turkey. The prevalence of HCV specific antibody ranges from 0.3 to 1.8 percent among blood donors[31]. As seen in this study, pediatric patients with cancer and hemoglobinopathies have high HBsAg seropositivity (15.5%) and anti HCV reactivity (11.5%). However, in this study patients with GBV-C RNA positivity had no co-infection with HCV or HBV. In addition, the rate of seroprevalence of GBV-C in Turkish patients receiving multiple transfusions was not higher than that in patients in western countries, where HBV and HCV infection are not common. We think that transmission of GBV-C may not be directly related to the transmission of HBV or HCV infection, or else the presence of GBV-C infection may be related to the other unknown factor.

In conclusion, it may be considered that pediatric patients with cancer and hemoglobinopathies who have had multiple transfusions in Turkey are at risk of being infected with GBV-C, in addition to HBV and HCV. In these patients, GBV-C infection may cause chronic hepatitis characterized by elevated serum ALT without co-infection with HBV or HCV. For this reason, the development of simple and reliable tests to detect HGV infection are needed.

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