

Full Length Research Paper

A study on the serologic and molecular prevalence of hepatitis G virus (HGV) and hepatitis C virus (HCV) infections in patients with thalassemia in Larestan of Iran

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Hepatitis G virus (HGV) and hepatitis C virus (HCV) infections may have role in introducing or complicating the clinical outcome in patients with thalassemia. In this study, the molecular and serological prevalence of HGV and HCV infections were evaluated in patients with thalassemia and compared with healthy controls. In a cross sectional study, the samples were collected from 86 patients with thalassemia and 100 healthy controls. The anti-HCV and HGV antibodies were evaluated by enzyme-linked immunosorbent assay (ELISA) methods. Also, the HGV and HCV viremia were analyzed in patients with thalassemia and controls by multiplex-nested-real time-polymerase chain reaction (RT-PCR) protocol. Our results showed that the anti-E2-HGV and HCV antibodies were found in 16 of 86 and 18 of 86 of thalassemia patients and none of controls, respectively. HGV viremia was diagnosed in 13 of 86 of patients with thalassemia and in 1 of 100 of controls. HCV-RNA was diagnosed in 12 of 86 and 6 of 100 of thalassemia and healthy controls, respectively. The diagnosis of significant higher prevalence of HGV and HCV in patients with thalassemia compared with controls in this region of Iran emphasized the importance of these lymphotropic viral hepatitis infections in pathogenesis and outcome of thalassemia patients.

Key words: Lymphotropic viruses, hepatitis G virus (HGV), hepatitis C virus (HCV), thalassemia.

INTRODUCTION

β -Thalassemia or thalassemia major is the most common hereditary blood disorder in the Mediterranean basin especially in Iran that has affected 25,000 individuals and has 4 to 5% prenatal carrier rate in total population (Mirmomen and Alavian, 2005; Rahimi et al., 2008; Akhavan-Niaki et al., 2011). Multi-transfusion in patients with thalassemia necessary for patient survival is at high risk for transfusion-associated infectious diseases such

as hepatitis viruses. Prevalence of viral hepatitis infections in thalassemic patients is related to the prevalence of the infections in the donor population and screening test technology used for blood and blood products (Ocak et al., 2006; Al-Sheyab et al., 2001). Preventing transmission of these blood-borne viruses remains a major preoccupation for transfusion services worldwide (Ocak and Kaya, 2006). Hepatitis viruses and lymphotropic flaviviruses, including hepatitis C and G viruses (HCV and HGV) may have important role in pathophysiology of Thalassemia.

According to the earlier reports, HGV RNA was detected in 12.9% of patients with thalassemia, which is

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similar to 11% reported by Cacopardo et al. (1998). Moreover, HGV genome has higher prevalence (19%) in serum of Greek thalassemic patients (Amini et al., 2005). In Taiwanese population, HGV viremia was significantly found in 1.7 vs. 18% and 1 vs. 14% of normal blood donors compared with patients with thalassemia (Kao et al., 1997; Linnen et al., 1996; Chung et al., 1997). For the potential of simultaneous transmission of hepatitis viruses, the co-infection of HGV and HCV was found in 25% of thalassemia patients (Amini et al., 2005; Tagariello et al., 1996). Between 0.1 to 1% of Iranian normal blood donors were infected by HCV. The prevalence of HCV infection in thalassemia patients was significantly higher. Reports from different geographical regions of Iran re-confirmed this hypothesis by obtaining HCV infection in 19.3 to 64% of patients with thalassemia. (Mirmomen et al., 2006; Hassanshahi et al., 2011; Martin and Fabrizi, 2008; Fissell et al., 2004; Laosombat et al., 1997; Boroujerdnia et al., 2009; Ansar and Kooloobandi, 2002; Karimi and Ghavanini, 2001; Tamaddoni et al., 2007).

Therefore, based on earlier studies and due to the little data available on the prevalence of hepatitis viruses especially HGV infection in patients with thalassemia in Larestan, the molecular prevalence of HGV and HCV infection was evaluated.

MATERIALS AND METHODS

The ethylenediaminetetraacetic acid (EDTA) treated blood samples were collected from two studied groups, including 86 patients with thalassemia who multi-transfused in Larestan Blood Transfusion Center and 100 healthy control group who clinically and laboratory ruled out any hematological abnormalities between year 2008 to 2010. The molecular and serological prevalence of HGV and HCV infections were analyzed in patients with thalassemia and compared with healthy controls. Also, some possible risk factors of thalassemia pathogenesis, including age, gender, marriage, and history of smoking, transfusion, surgery, bone marrow transplantation and HIV infection were statistically analyzed for all studied patients with non-Hodgkin's lymphomas (NHL).

HGV and HCV serological analysis

Antibody against E2 glycoprotein of HGV (Anti-E2 Ab) was evaluated in plasma samples by third generation ELISA kit (DIAPRO, Italy and Dade Behring Marburg, Germany), according to the manufacturer's instructions. Anti-HCV antibody (HCVAb) was also evaluated in the studied plasma samples by third generation ELISA kit (DIAPRO, Italy), according to manufacturer's instruction.

HCV-RNA extraction and amplification

The HGV and HCV-RNA genomes were extracted from plasma samples by RNX plus extraction procedure as previously described (Kao et al., 1997). The quality of extraction technique was evaluated by spiking of HGV and HCV-RNA in negative plasma samples.

HGV and HCV cDNA synthesis

The presentation of HGV and HCV-RNA genomes was analyzed by an in-house HGV/HCV-nested-real time-polymerase chain reaction (RT-PCR) protocol. First, cDNA was sensitized from HGV and HCV genomes by in-house protocol as follow: 3 μ L of viral extracted RNA was incubated at 25°C for 1 h and at 72°C for 10 min after treating with random hexamer and Moloney murine leukemia virus reverse transcriptase (M-MuLV-RT). The 20 μ L RT-master mix contained 0.2 mmol of dNTPs, 0.01 mg/ml of random hexamer, 7.5 U/ml of M-MuLV-RT, 1 U/ml of ribonuclease inhibitor, and 4 μ L of 5 μ L RT- buffers.

HGV and HCV nested-PCR protocol

The PCR master mix of simple PCR contained 2 μ L of cDNA, 0.1 pmol/ μ L of primers, 0.2 mmol of dNTPs, 2.5 U of Taq DNA polymerase, 2.5 μ L of 10X PCR buffer, and 1.5 mmol of MgCl₂. The 2 μ L of simple PCR product was used in nested PCR step with the same condition as simple PCR mix. The total volume per reaction in the two rounds of simple and nested PCR protocol was 25 μ L. The thermocycling conditions for simple and nested PCR rounds were the same. Simple PCR was initiated with a first round at 95°C for 5 min, followed by a second round of 25 cycles at 94°C for 50 s, 55°C for 40 s, and 72°C for 50 s, and finalized with extension at 72°C for 3 min. The nested PCR protocol was initiated with a first round at 95°C for 5 min followed by a second round of 35 cycles at 94°C for 40 s, 64°C for 35 s, and 72°C for 40 s, and finalized with extension at 72°C for 3 min.

Statistical analysis

Significant differences of serological and molecular diagnostic markers of studied HGV and HCV between thalassemia patients and controls and also statistical correlations between viral hepatitis diagnostic indices and possible risk factors of thalassemia were analyzed by use of parametric and non parametric analyses with SPSS for Windows (version 15, Chicago, IL, USA). A level of $p \leq 0.05$ was accepted as statistically significant.

RESULTS

In total, 41 of 86 (47.7%) patients with thalassemia were male and 45 of 86 (52.3%) of patients were female. Also 39 of 100 (39%) of controls were male and rest of them 61 of 100 (61%) were female with average age of 36 years old.

Molecular presentation of HGV and HCV

HGV-RNA was detected in 13 of 86 (15.11%) of patients with thalassemia. HGV-RNA was also diagnosed in 1 of 100 (1%) of control group. Significant higher odds of HGV viremia was found among patients with thalassemia ($p=0.0001$, OR=0.057, 95% CI=0.007 - 0.443) (Table 1). On the other hand, HCV-RNA was diagnosed in 12 of 86 (13.95%) and 6 of 100 (6%) of thalassemia and healthy controls, respectively. Also, significant higher odds of

Table 1. The prevalence of HGV and HCV infective markers in patients with thalassemia and controls.

| Markers | NHL patients no. (%) | Control group no. (%) | P value | OR | 95% CI |
|---------|----------------------|-----------------------|---------|-------|---------------|
| HCV Ab | | | | | |
| +Ve | 18/86 (20.9) | 0/100 (0) | 0.0001 | 0.791 | 0.702 - 0.882 |
| -Ve | 68/70 (65.1) | 100/100 (100) | | | |
| HGV Ab | | | | | |
| +Ve | 16/86 (16.6) | 0/100 (0) | 0.0001 | 0.814 | 0.736 - 0.900 |
| -Ve | 80/86 (69.4) | 100/100 (100) | | | |
| HGV-RNA | | | | | |
| +Ve | 13/86 (15.1) | 1/100 (1) | 0.0001 | 0.057 | 0.007 - 0.443 |
| -Ve | 73/86 (84.9) | 99/100 (99) | | | |
| HCV-RNA | | | | | |
| +Ve | 12/86 (13.9) | 94/100 (94) | 0.05 | 0.394 | 0.141 - 1.098 |
| -Ve | 74/86 (86.1) | 6/100 (6) | | | |

+Ve, Positive; -Ve, negative; NV, not valid.

HCV viremia was observed among patients with thalassemia ($p=0.05$, $OR=0.394$, $95\% CI=0.141-1.098$) (Table 1). Additionally, 6 of 13 HGV infected patients with thalassemia HCV-RNA were simultaneously found.

Serological presentation of HGV and HCV

Anti-E2- HGVAbs were found in 16 of 86 (16.60%) of thalassemia patients and none of controls. HCVAbs were found in 18 of 86 (20.93%) of thalassemia patients and none of control group (Table 1). Significant higher odds of positive HGVAbs ($p=0.0001$, $OR=0.814$, $95\% CI=0.736-0.900$) and positive HCVAbs ($p=0.0001$, $OR=0.791$, $95\% CI=0.702-0.882$), respectively were found among patients with thalassemia (Table 1).

Single and multiple infections of HGV and HCV

At least one of molecular and immunological markers of HGV and HCV was found in 23 of 86 (26.7%), and 28 of 86 (32.5%) of patients with thalassemia, respectively. In addition, HGV and HCV infective markers were found in 1 of 100 (1%), and 6 of 100 (6%) controls, respectively. Co-infection of HGV and HCV was diagnosed by evaluation of different markers of these two viruses in 11 of 86 (12.8%) of patients with thalassemia. Co-infection of HGV with HCV was not diagnosed in any controls.

HGV and HCV and risk factors of thalassemia

Significant higher odds of positive anti-E2 HGVAbs

($p=0.045$), positive HCV-RT-PCR ($p=0.0001$), and positive HGV-RT-PCR ($p=0.0001$) was observed with age. Significant higher odds of a positive HGVAbs ($p=0.011$, $OR=0.239$, $95\% CI=0.074-0.772$), positive HCV-RT-PCR ($p=0.0001$, $OR=0.038$, $95\% CI=0.005-0.293$), and positive HGV-RT-PCR ($p=0.0001$, $OR=0.053$, $95\% CI=0.007-0.413$) was observed among males with thalassemia. Significant higher odds of a positive HCVAbs was not associated with age ($p=0.936$) and among males or females ($p=0.405$, $OR=0.787$, $95\% CI=0.298-2.083$). The times of receiving blood were found in thalassemia patients with significant higher odds of positive HCVAbs ($p=0.0001$) and positive HGVAbs ($p=0.0001$). However, the significant higher odds of the viremia of HGV ($p=0.08$) and HCV ($p=0.337$) was not found with times of blood transfusion in studied patients (Table 2).

DISCUSSION

Iran is located in the thalassemia belt and about 10% of the population suffers from this hereditary hematological abnormality (Haghpanah et al., 2010). Particularly in Larestan, which is located in southern of Iran, the prevalence of this disorder is very high as a result of the close relative marriage. Some of blood-borne viruses are common and have determinative role in outcome of patients with thalassemia. Viral hepatitis infections, including HGV and especially HCV and their related hepatic and extrahepatic clinical manifestations are at the higher risk of interfering in the pathophysiology of thalassemia (Mirmomen and Alavian, 2005; Boroujerdnia et al., 2009). Therefore, in this study, the molecular and serological prevalence of hepatitis viruses was evaluated

Table 2. Relationship between HGV and HCV-RNA infected patients with thalassemia and the number of blood transfusions.

| Blood transfusion received | No. patients | Age (year \pm S.D) | Sex | | HGV-RNA (%) | | HCV-RNA (%) | |
|----------------------------|--------------|----------------------|------|--------|--------------|--------------|--------------|--------------|
| | | | Male | Female | No. positive | No. negative | No. positive | No. negative |
| 2 - 15 | 10 | 8.6 \pm 2.95 | 6 | 4 | 1 (10) | 9 (90) | 2 (20) | 8 (80) |
| 16 - 40 | 16 | 13.81 \pm 5.20 | 9 | 7 | 6 (37.5) | 10 (62.5) | 3 (18.75) | 13 (81.25) |
| 41 - 80 | 30 | 12.16 \pm 4.17 | 11 | 19 | 4 (13.33) | 26 (86.66) | 4 (13.33) | 26 (86.66) |
| 81 - 100 | 20 | 16.95 \pm 4.67 | 9 | 11 | 1 (5) | 19 (95) | 2 (10) | 18 (90) |
| >100 | 10 | 17.4 \pm 3.68 | 6 | 4 | 1 (10) | 9 (90) | 1 (10) | 9 (90) |
| Total | 86 | 13.77 \pm 5.09 | 41 | 45 | 13 (15.11) | 73 (84.88) | 12 (15.11) | 13 (15.11) |

in patients with thalassemia compared with healthy controls.

Based on earlier reports, HCV infection has higher prevalence compared with normal blood donors worldwide. In Iran, <1% of normal blood donors are infected by HCV. However, reports from different geographical regions of this country have shown significant higher prevalence of anti-HCV antibody in thalassemia patients (Mirmomen et al., 2006; Hassanshahi et al., 2011; Martin and Fabrizi, 2008; Fissell et al., 2004; Laosombat et al., 1997; Boroujerdnia et al., 2009; Ansar and Kooloobandi, 2002; Karimi and Ghavanini, 2001; Tamaddoni et al., 2007). Ansar and Kooloobandi, (2002) from North of Iran reported that anti-HCV antibody was found in 63.8% of thalassemia patients (Ansar and Kooloobandi, 2002). In another study on Iranian thalassemic patients, 24.2% of them were found to have anti-HCV antibody (Alavian et al., 2005). Boroujerdnia et al. (2009) also revealed the prevalence of anti-HCV antibody in 28.1% of thalassemia patients in Khuzestan province in southwest of Iran (Boroujerdnia et al., 2009).

In this study, history of HCV-infection including anti-HCV antibody and HCV viremia was also diagnosed significantly higher in thalassemia patients. HCV-RNA was diagnosed in 12 of 86

(13.95%) of patients with thalassemia vs. 6 of 100 (6%) controls. In addition, 6 of 13 HGV infected patients with thalassemia HCV-RNA were simultaneously found. Significant higher odds of positive HCVAb ($p=0.0001$) was also found among patients with thalassemia (Table 1), as well as significant higher odds of positive HCV-RT-PCR ($p=0.0001$) with age. Significant higher odds of a positive HCV-RT-PCR ($p=0.0001$) was observed among males with thalassemia. Significant higher odds of a positive HCVAb was not associated with age ($p=0.936$) and among males or females ($p=0.405$). The times of blood transfusion was found in thalassemia patients with significant higher odds of positive HCVAb ($p=0.0001$), but not HCV viremia ($p=0.337$) (Table 2). In other studies, the impact of HCV infection in patients with major thalassemia was as follow: Hassanshahi et al. (2011) found anti-HCV antibody in 81 of 181 (44.7%) patients suffering from major thalassemia; Karimi and Ghavanini, (2001) diagnosed anti-HCV antibody in 15.7% thalassemic children with a history of multiple transfusions (Karimi and Ghavanini, 2001). In other study, 10.6% of thalassemia cases were positive for anti-HCV antibody (Tamaddoni et al., 2007). Mirmomen et al. (2006) found history of HCV infection in 19.6% of β -thalassemic patients.

One hundred and forty-one of 732 (19.3%) patients were anti-HCV antibody positive. Univariate analysis showed that thalassemia major ($p= 0.01$), older age ($p= 0.001$), longer duration of transfusion ($p= 0.0001$), and higher serum ferritin level ($p= 0.002$) were significantly associated with a higher seroprevalence of HCV (Mirmomen et al., 2006). In Arabian neighboring countries of Iran, HCV infection was found in 33% of Kuwait and 40% of Bahrain and Jordan patients with thalassemia (Al-Fuzae et al., 1998; Al-Mahroos and Ebrahim, 1995). Also in Turkey, it was estimated that the frequency of thalassemia trait varies from 1.4 to 10.8% (with average 2%) and the HCV seroprevalence was 4.5% in patients with thalassemia (Ocak et al., 2006).

Recently, HGV which has no determined pattern of pathogenesis and clinical outcome in blood disorder was focused to evaluate in thalassemia patients. Moreover, there is little data on the prevalence of HGV in thalassemia patients in Iran. In earlier studies, the significantly higher prevalence of HGV viremia was found in blood donors versus patients with multi-transfused anaemia (1.7 vs. 18%) and versus poly-transfused Taiwanese children, respectively (Kao et al., 1997; Linnen et al., 1996; Chung et al., 1997). HGV RNA was also detected in 12.9% of Iranian

patients with thalassemia, which is similar to Italian patients (11%) and Greek patients with thalassemia (19%) (Cacopardo et al., 1998; Amini et al., 2005). But in other study, the HGV viremia was found in 9 of 402 (2.24%) multi-transfused thalassemia cases. In this report, HGV genome was significantly found in patients with thalassemia and controls (15.11 vs. 1%), respectively (Table 1). Significant higher odds of HGV viremia was found among patients with thalassemia ($p=0.0001$, $OR=0.057$, $95\% CI=0.007-0.443$) (Table 1). Also, Anti-E2 antibody was significantly higher prevalence in thalassemia patients versus controls (16.60% vs. 0%) (Table 1). Significant higher odds of positive HGVAb ($p=0.0001$) was found among patients with thalassemia (Table 1). In addition, significant higher odds of positive HGVAb ($p=0.045$) and positive HGV-RT-PCR ($p=0.0001$) was observed with age. Significant higher odds of a positive HGVAb ($p=0.011$) and positive HGV-RT-PCR ($p=0.0001$) was found among males with thalassemia. The times of blood transfusion was found in thalassemia patients with significant higher odds of positive HGVAb ($p=0.0001$), and significant higher odds of the viremia of HCV ($p=0.337$) was not found with times of blood transfusion in studied patients (Table 2).

On the other hand, for familial relationships between HGV and HCV has been suggested that HCV and HGV are transmitted together (Cacopardo et al., 1998). In previous investigations, high prevalence of HGV and HCV co-infection was prevalent in patients with thalassemia (25%) (Amini et al., 2005; Desai et al., 2004). In this study, the co-infection of HGV and HCV was diagnosed in 12.8% of patients with thalassemia, but this co-infection was not seen in any controls. Finally, diagnosis of significant higher serological and molecular prevalence of single and co-infection of HGV and HCV in patients with thalassemia in Larestan of Iran, emphasized more on the importance of these lymphotropic viral hepatitis infections in introducing and complicating the clinical outcome of thalassemia patients.

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