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Detection of Occult Hepatitis C Virus Infection (OCI) in Bone Marrow Mononuclear Cells

Mohamed Darwish Ahmed Abd Alla[1], Yasser Mohamed Mohamed El-Dessouky[1], Khaled Abdelmoamen Ahmed Ali[2], Moukhtar Ragab Ramadan[3], Raefat Mohamed Abd El Fattah[2], Abdel Ghany Abdel Sattar Aish[4], and Mostafa Kamel El Awady[5]

[1]Department of hepatology, Gastroenterology and Infectious Diseases, [3]Radiology Department, Faculty of Medicine, Al-Azhar University; [2]Nasser Institute for Research and Treatment; [4]Shebin El-Koum Teaching Hospital; [5]Department of Microbial Biotechnology, National Research Center, Cairo, Egypt.

Correspondence to: Mohamed Darwish Ahmed Abd Alla, Tel: +2 0109-417-5209, E-mail: darwish0716@azhar.edu.eg

ABSTRACT

Background and aim: Occult HCV infection (OCI) diagnosis by cellular-PCR encourages further research for more understanding of extrahepatic tissues affection during non-viremias. OCI among Egyptians recruits more attention because of the convenient diagnostic tools and the availability of direct acting antivirals (DAAs). Our study aims to recognize HCV-infection of bone marrow mononuclear cells (BMMCs) in antiviral IgG-antibody seropositive non-viremic subjects.

Subjects and Methodology: Our case control study included 70 subjects who were divided into three groups: - ***I***: non-viremic IgG-seronegative controls (n=25), ***II***: naïve HCV-RNA viremic IgG-seropositive controls (n=15), ***III***: naïve non-viremic IgG-seropositive patients (n=30). Sera of all study subjects were screened for anti-HCV IgG-antibodies by ELISA and for HCV-RNA by PCR. BMMCs and peripheral blood mononuclear cells (PBMCs) of all study populations were tested for HCV-RNA infections by cellular-PCR.

Results and conclusions: HCV-RNA viremias were seen when bone-marrow is functioning (P<0.05), while non-viremias were common in bone-marrow failure (P<0.05). There was 100% concordant positivity between serum and cellular HCV-RNA-PCRs in viremic patients. HCV-BMMCs and PBMCs PCRs respectively added extra 20% (P=0.024) and 13.33% (P=0.119) as newly diagnosed infections compared to none by serum-PCR in IgG-antibody seropositive non-viremic subjects. Respectively, non-viremic population (n=55) showed 14.29% and 9.1% positive intra-mononuclear cells HCV-RNA infection in bone marrow (P=0.002) and peripheral blood (P=0.057) compared to serum PCR. In conclusion, consecutive screening of peripheral blood and bone marrow mononuclear cells for HCV-RNA detection by cellular-PCR recognizes naïve OCI in IgG-seropositive non-viremic patients. Functioning bone marrow would be required for persistence of HCV-RNA viremia.

Keywords: Occult HCV Infection, Bone Marrow and Peripheral Blood Mononuclear cell-PCRs.

INTRODUCTION

Management of occult HCV infection (OCI) in naïve and experienced patients has been questionable because in addition to diagnostic difficulties, the fates of treatment trials haven't been thoroughly evaluated. Before availability of the noninvasive peripheral blood mononuclear cells (PBMCs) PCR [(1)], diagnosis of OCI required hepatocyte testing for intracellular HCV-RNA existence [(2)]. The use of PBMCs-PCR in detecting intracellular HCV-RNA genomic materials is specific with high negative predictive values, however its sensitivity is around 60% in diagnosing cryptogenic hepatitis C virus infection [(2)]. Consistency and relationship between intra-PBMCs and intra-bone marrow mononuclear cells (BMMCs) HCV-RNA detection has been unveiled. Despite the documented HCV hepatotropism, isolation of HCV-RNA from extra-hepatocytes sources was successful [(3)].

Replications of genomic HCV-RNA inside PBMCs from naïve and posttreatment patients were found to be followed by overt viremia after disappearance of serum virus particles [(4,5)]. Therefore, serum and cellular testing for extracellular and intra-PBMCs HCV-RNA genomic materials, in spontaneous and post-therapy disappearance of viral particles in non-viremias, would confirm concordant disappearance of the virus from serum and cells [(6,7)]. Despite the reported 90% post-DAAs therapy SVR, only serologic relapse was considered [(8)]. No enough attention was paid to posttreatment intracellular persistent or relapse of viral-RNA infection that leads to serologic relapse with progressive hepatic damage [(4,5)], and global aggravation of HCV health problem worldwide [(9)]. More comprehensive investigations and new therapeutic protocols should be proposed to continue management of HCV infection beyond the false clinical recovery [(10-13)]. In 2018, Abd Alla et al. evaluated post-DAAs therapy outcomes before end of treatment (EOT); they extended DAAs therapy beyond 12 weeks in those who had intra-PBMCs HCV-RNA genomic materials till the end of 24th week. The intracellular HCV-RNA was cleared in most cases and SVR was improved [(7)].

The relationship between HCV infection and bone marrow disease syndrome was reported in essential mixed cryoglobulinemia with lymphoproliferative disorders, non-Hodgkin's lymphoma and other hematological malignancies [(14-16)]. It was reported that association may be related to HCV genotypes variations in different parts of the world, however regional and racial factors may play a

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considerable role [17]. In OCI, none Hodgkin lymphoma of the liver or salivary glands showed 50-fold elevated risk and 10% of type II mixed cryoglobulinemia, but not to multiple myeloma, can be transformed to malignant lymphoma years after diagnosis [18]. An association between OCI and multiple myeloma (MM) was proposed, but not documented [19]. On the other hands, discordance of OCI with B cell lymphoma was also reported [20].

Infection of mononuclear cells with HCV may occur outside bone marrow, in peripheral blood, during RNA viremia (extra-marrow infections). It may also happen in bone marrow itself because of viral infection of bone marrow stem tissues (intra-marrow infection) during exposure to the virus for the first time. PBMC life span in host circulation as well as efficiency of bone marrow productivity seem to play a major role in persistent intra-mononuclear HCV infection as well as viremia. Accordingly, the current study was designed to evaluate OCI in BMMCs of patients presented with positive serum anti-HCV IgG-antibodies, but negative serum SRT-PCR.

PATIENTS AND METHODS

A. Study populations

The current study was conducted at the Department of Hepatology, Gastroenterology, and Infectious diseases at Al-Hussein University Hospital, in addition to Hematology and Bone Marrow Transplant unit at Nasser Institute. Subjects of our research consisted of selected 70 Egyptian subjects who were divided into the following three groups:

a) ***Group I (negative control)***: consisted of 25 subjects who have negative serum anti-HCV IgG antibodies. They were clinically free recruits for bone marrow donation (volunteers)

b) ***Group II (Positive control group)***: included 15 naïve patients with positive serum anti-HCV IgG antibodies and HCV single step reverse transcription (SRT)-PCR. Patients in this group were not submitted to the antiviral therapy at any point of time in their life.

c) ***Group III (targeted group)***: contained 30 naïve patients presented with positive serum anti-HCV IgG antibodies and negative HCV serum SRT-PCR.

Age range of the selected subjects was between 18 and 70 years with matching age and gender in all study groups. All subjects were submitted to full clinical exam, routine lab investigations, routine image screening as well as the below-mentioned specific procedures. Exclusion criteria included pregnant woman; other causes of chronic liver diseases (e.g. autoimmune hepatitis, biliary cirrhosis, cardiac cirrhosis); patients suffering from hepatocellular carcinoma, patients suffering from chronic uncontrolled debilitating disease e.g. uncontrolled diabetes mellitus, sarcoidosis, SLE; cases presented after DAAs treatment; patients with recent blood transfusion or on intravenous drug abuse; patients receiving immunosuppressive or cytotoxic medicine; and finally, patients presented with decompensated hepatic disorders.

B. ELISA for detection of anti-HCV IgG antibodies

Indirect ELISA method for detection of antibodies to HCV in two-step incubation procedure was performed as originally described [21].

C. Serum HCV SRT-PCR

Serum HCV-RNA extraction and single step reverse transcription PCR (SRT-PCR) procedure, internal control of the extracted RNA and/or contamination, and HCV-RNA quantification were done as described by [22].

D. Bone marrow aspirates or biopsy

Each subject was submitted to duplicate of bone marrow aspiration or biopsy at baseline of the study. Repeating the same procedure is indicated only in cases of failure. The aspiration was done after appropriate local anesthesia by advancing a specially designed 14- to 16-gauge needle fitted with an obturator through the cortex into the medullary space. After the obturator removal, marrow was aspirated using a syringe and negative pressure. Individual marrow particles were spread on a glass slide and stained with Romanowsky stain to evaluate its content and the subsequent procedure success [23]. The bone marrow trephine biopsy was performed on bone marrow aspiration failure by using a larger needle (Jamshidi needle) which can cut a cylinder of bone from the medullary space [24]. Aspirate samples or biopsy contents for PCR were collected into EDTA tubes. BM mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Biotech (density gradient centrifugation) and were used immediately [25].

E. Cellular HCV RNA-PCR

a) Extraction of RNA from BMMCs:

After isolation of mononuclear cells from marrow aspirate samples or biopsies, RNA was extracted and submitted to PCR as described below in peripheral blood mononuclear cells management.

b) Extraction of RNA from PBMCs:

Peripheral blood (200 µl) was diluted with 10 ml freshly prepared red blood cell alkaline buffer (38.8 mmol/L NH_4Cl, 2.5 mmol/L K_2HCO_3, 1 mmol/L EDTA, pH 8.0). After 10 minutes incubation at room temperature, nucleated cells were washed with the same buffer, the cells were suspended in 500 *µl* anti nuclease solution (4 mol/L guanidinium isothiocyanate containing 25 *mmol*/L sodium citrates, 0.5% and 0.1 *mol*/L ß- mercaptoethanol). A single step method described by [26].

c) Amplification of HCV-RNA by nested RT-PCR in PBMC`s:

Qualitative detection of HCV RNA by reverse transcription- polymerase chain reaction was done according to [27] with few modifications [22].

e. Statistics

Recorded data were analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data were expressed as mean± standard deviation (SD). Qualitative data were expressed as frequency and percentage. Independent-samples t-test of significance was used when comparing between two means. Chi-square (x^2) test of significance was used to compare proportions between two qualitative parameters. Pearson chi-square and likelihood-ratio chi-square, Fisher's exact test and Yates' corrected chi-square were computed for 2x2 tables. The confidence interval was set to 95% and the margin of error accepted was set to 5%. The p-value was considered significant at P-value <0.05.

Written informed consent:

An approval of the study was obtained from Al- Azhar University academic and ethical committee. Every patient signed an informed written consent to participate in the current study.

- **Conflict of Interest:** The authors have no conflict of interest related to this publication.

RESULTS

I. Histopathologic diagnosis of bone marrow aspirates in the studied populations

Table 1 categorized various histopathologic diagnosis of the studied populations into 10 conditions. Data in the same table indicated that none of the patients presented with AA, MDS, HS, leukemia, MF, and metastasis had positive serum HCV-RNA PCR (100% were non-viremia), despite the recognized levels of anti-HCV IgG antibodies, compared to viremic populations as identified by the P values in the table. The bone marrow histopathologic diagnosis of most of the previously mentioned lesions (AA, MDS, leukemia, MF, and metastasis) are associated with none functioning hematopoietic tissues. On the other hands, the histopathologic bone marrow features that identified marrow tissues activities are associated with significant viremia as in **anemia** and **normal bone marrow looking** compared to non-viremic. It seems that functioning marrow activities is one of the main requirements of HCV-RNA viremia to become true.

Table 1: Distribution of bone marrow aspiration histopathologic diagnosis among non-viremic IgG seropositive and viremic patients

	Group II (N=15)	Group III (N=30)	values
A.A.	0.0 (0.0%)	7.0 (23.33%)	**0.023**
MDS	0.0 (0.0%)	6.0 (20.0%)	**0.037**
MM	1.0 (6.67%)	0.0 (0.0%)	0.167
HS	0.0 (0.0%)	3.0 (10.0%)	0.144
Leukemia	0.0 (0.0%)	7.0 (23.33%)	**0.023**
Lymphoma	1.0 (6.67%)	4.0 (13.33%)	0.285
MF	0.0 (0.0%)	1.0 (3.33%)	0.334
Metastasis	0.0 (0.0%)	1.0 (3.33%)	0.334
Anemia	8.0 (53.33%)	0.0 (0.0%)	**0.001**
Normal	5.0 (27.78%)	1.0 (3.33%)	**0.007**

Mid P exact 1 tailed P values showed frequent recognition of anemia and normal bone marrow in viremic patients (group II) (P<0.05) compared to non-viremic population in group III. In addition, aplastic anemia, myelodysplastic sclerosis, and leukemia were found more often in non-viremic IgG seropositive group III (P<0.05) compared to viremic patients. AA, aplastic anemia; MDS, myelodysplastic syndrome; MM, multiple myelomatosis; HS, hypersplenism; MF, Myelofibrosis.

II. Comparison of BMMCs and PBMCs PCRS results with serum SRT-PCR in the studied population.

The relationship between serum and cellular HCV-PCR was demonstrated in table 2. There was 100% concordance between serum and both kind of cellular HCV-PCR in group II. The data set in the same table reported that positive serum HCV-RNA PCR is significantly associated with intracellular viral infection in viremic patients compared to non-viremic subjects in both group I and III. Despite the insignificant difference, the currently presented data of non-viremic study populations showed higher frequency of: a) combined BMMCs and PBMCs b) solitary BMMCs c) solitary PBMCs, HCV-RNA positive PCR in the presence of anti-viral IgG antibody positive serology in group III compared to the seronegative group I.

Table 2: Concordance of HCV-RNA detection in mononuclear cells by cellular PCR and in serum by SRT-PCR in all study populations

Groups of study populations	Subjects with positive intra-mononuclear cells HCV-RNA PCR		
	BMMCs + PBMCs	Solitary BMMCs	Solitary PBMCs
A. Group I (n=25)	1 (4.4%)	1 (4.0%)	0.0 (0.0%)
B. Group II (n=15)	15.0 (100%)	0.0 (0.0%)	0.0 (0.0%)
C. Group III (n=30)	3 (10.0%)	3 (10.0%)	1 (3.33%)
P value: A vs B	<0.001	0.313	0.334
A vs C	0.23	0.23	0.273
B vs C	<0.001	0.144	0.334

In group II, the concordance between serum and cellular HCV-PCR was 100%. The mid P exact 1 tailed P values indicated that serum HCV-RNA PCR was significantly associated with intra-cellular viral infection in viremic patients compared to non-viremic subjects in both group I and III (P=0.000001). Higher frequency of combined and solitary

cellular (BMMCs and / or PBMCs) HCV-RNA positive PCR was associated with presence of anti-HCV IgG antibody positive serology in group III compared to the seronegative group I, despite the insignificant changes (P>0.05).

III. Results of intracellular HCV-RNA detection by BMMCs and PBMCs PCRs in subjects presented with negative serum SRT-PCR

Table 3 identified productivity of intracellular detection of HCV RNA by cellular PCR procedure when serum SRT-PCR is negative. The data showed a significant frequency of intracellular HCV-RNA detection by both BMMCs (P=0.002) and PBMCs (P=0.03) PCRs in those who presented with negative viral serum PCR. On the other hands, comparing results of BMMCs with PBMCs PCRs (8 out of 55 with 4 out of 55) showed insignificant difference (P=0.121) in the same study population.

Table 3: Comparison of solitary intracellular HCV-RNA detection by BMMCs and PBMCs PCR in non-viremic study subjects

Type of cellular HCV-PCR	HCV RNA serum SRT-PCR negative populations (n=55)	
BMMCs	8 (14.545%)	0 (0.0%)
PBMCs	0 (0.0%)	4 (7.273%)
Mid P exact 1 tailed P values	0.002	0.03

The data presented in the current table showed significantly increased frequency of intracellular HCV-RNA detection by both BMMCs (P=0.001486) and PBMCs (P=0.029538) PCRs in those who presented with negative viral serum PCR. Contrarily, no significant change was observed on comparing BMMCs and PBMCs PCRs (8 out of 55 with 4 out of 55: P=0.120801) in the same study population

DISCUSSION

The current research reported detection of occult HCV infection (OCI) in both IgG antibody seropositive and seronegative non-viremic subjects by bone marrow mononuclear cells (BMMCs) and peripheral blood mononuclear cells (PBMCs) PCRs. Both serum SRT- PCR and anti-HCV IgG antibody ELISA were used to select the current study populations and to group them into three groups; group I (negative controls), group II (positive controls), and group III (targeted population).

Data of the current research indicated that HCV-RNA viremias were associated with functioning bone marrow, and the non-viremic populations had non-productive bone marrow tissues. In addition, HCV viremia is always associated with positive mononuclear cell PCR of either bone marrow or peripheral blood. Respectively, HCV BMMCs-PCR added extra 20% (6 out of 30) and 8% (2 out of 25) as newly diagnosed positive cases in serum anti-HCV IgG seropositive and seronegative cases. On the other hands, HCV PBMCs-PCR showed respectively positive results in 13.33% (4 out of 30) and 4% (1 out of 25) in serum anti-HCV IgG seropositive and seronegative subjects who were non-viremic. Our study recommended consecutive testing of PBMCS and BMMCs by cellular-PCR to diagnose OCI in naïve IgG-antibody seropositive patients with negative serum SRT-PCR. The same data set concluded that functional productivity of bone marrow tissues would be required for maintaining HCV-RNA viremia.

Until now, it is not known whether infection of PBMCs with HCV occurs anytime in peripheral blood during RNA viremia (extra-marrow infections); or because of viral infection of mononuclear cells precursors e.g. mother and daughter stem cells within bone marrow tissues (intra- marrow infection) during first exposure to the virus regardless to viremia. The life span of PBMC seems to be too short to allow full establishment of intracellular HCV infection only during direct contact with viral-RNA in host blood vessels for days during viremia. Even after elimination of viremia, the contact between viral RNA and PBMCs in patient's circulation does not exist despite continuous detection of the intra-PBMCs virus genomic materials [(1-7)]. So, extra-marrow infection of PBMCs with HCV is questionable and may requires further research. On the other hands, intra-marrow infection is expected to last longer and is supposed to be associated with a better chance to get HCV-RNA well settled inside BMMCs precursors before release from marrow sources. This might explain the continuous intracellular infections with HCV-RNA in non-viremic patients. Based upon the above-mentioned assumptions, our study was designed to retrieve extra cases presented with OCI and have solitary BMMCs infection with HCV in non-viremic IgG seropositive patients.

The current study is a preliminary one that was dealing with the significance of using cellular HCV-PCR in detection of viral RNA genomic materials inside the recruited mononuclear cells from both bone marrow and peripheral blood. It is well established through several studies that detection of intracellular HCV-RNA genomic materials using different diagnostic procedures is considered as a solid prove of viral infection [(1-3)]. Intra-nucleated cellular detection of HCV-RNA genomic materials was done in hepatocytes, peripheral blood mononuclear cells, bone marrow mononuclear cells as well as other tissues by cellular PCR in addition to other solid diagnostic procedures like immunofluorescence. The clinical applications of tissue PCR in diagnosing infectious diseases has gone so far during the last few years. So, there are many clinically applied advantages of screening for HCV-intracellular infection when sera tested negative by SRT-PCR. Cryptogenic and Naïve IgG seronegative occult HCV infection were reported in few studies after diagnosis by cellular-PCR [(2, 28)].

The current study reported the same frequency of OCI in naïve subjects with no further evidence of HCV infection as reported by other researchers [(28)]. One of the major advantages of cellular HCV-PCR is more precise evaluation of posttreatment sustained virologic response (SVR), as elimination of virus from patient

serum doesn't indicate complete eradication [4,5]. In addition, cellular PCRs diagnose a major fraction of OCI in those who present with spontaneous disappearance of HCV from their blood at the same rate as reported in the current research [6]. Regardless to the kind of the infected nucleated cells with HCV, viral RNA extraction method is standardized, and the applied steps of PCR to the extracted nucleic acids are presented in a consistent manner. For all the above-mentioned advantages, our research team found that cellular PCR is an attractive method to evaluate and diagnose OCI. Various research groups have been using cellular PCR in diagnosing OCI and in posttreatment evaluation of HCV SVR through PBMCs screening [1,5-7,22,29]. Based on the above-mentioned discussion in the current paragraph, our study speculates that screening patients presented with anti-HCV IgG antibody negative serology for an expected occult or cryptogenic ongoing viral infection should be done on the following order: a) serum SRT-PCR: if negative proceed to b) PBMCs-PCR: if negative proceed to c) BMMCs-PCR.

The relationship between the hematopoietic activities of normal bone marrow tissues and peripheral blood cells constituents were established [30]. So, at the time of designing the current research, it was interesting to screen bone marrow nucleated cells for OCI before their release into systemic circulation. The infection of bone marrow hematopoietic tissues might be caused by different types of pathogens that find their way to blood stream of susceptible hosts. During HCV viremic stages, the free living viral genomic materials in patient circulation are expected to get hosted by nucleated cells within or outside bone marrow hematopoietic tissues with subsequent creation of new generations of infected mononuclear cells [31]. The infected mature bone marrow nucleated cells migrate through host circulation to hepatic and extra-hepatic tissues to participate in HCV disease syndrome. It seems that functioning bone marrow hematopoietic infected tissues is playing a predictable role in keeping up the viremic stage of HCV infections. This proposed assumption might explain the current study finding that reported HCV viremias were only found when host bone marrow tissues are normally functioning. Further research is required to verify adequacy of the above-mentioned proposed assumption. On the other hands, bone marrow tissue replacement or infiltration in different clinical situations is associated with dramatic changes in the picture of peripheral blood components. Histopathology of bone marrow aspirates or biopsies can recognize most of the systemic or localized disease syndromes that causes peripheral blood picture abnormalities [30]. Non-functioning bone marrow used to give-up the remaining hematopoietic activities to extra-bone marrow sites. The extra-bone marrow hematopoietic activities can be recognized in certain diseases like myelofibrosis, myelodysplastic sclerosis, aplastic anemia, and leukemia through examination of peripheral blood cellular constituents in addition to other specific tests [32]. The above-mentioned situations might help to assume that HCV infected PBMCs came from the extra bone marrow hematopoietic pre-infected tissues in cases of non-functioning bone marrow as reported in the above-mentioned diseases. More research projects should be scheduled to study roles of extra-bone marrow hematopoietic tissues infections in keeping up OCI particularly during bone marrow failure.

The concordance between detection of HCV-RNA genomic materials by PCR in serum and PBMCs was reported in many studies [5-7]. The current study results extended the previously mentioned concordance between HCV recognition in serum and in PBMCs to include bone marrow mononuclear cells. It is expected that the life span of mononuclear cells in the circulation is shorter than the virus life cycle inside any of the nucleated cells [33]. So, HCV infection of mononuclear cells would happen during genesis of these cells from an already infected source of stem tissues during hematopoiesis. This assumption was supported by a finding from the current study indicating that all HCV infected BMMCs are associated with positive intra-PBMCs co-infection when bone marrow is functioning. Further support came from the fact that solitary PBMCs HCV infection was addressed in patients presented with non-functioning bone marrow. The source of PBMCs in cases of non-functioning bone marrow is expected to be the extra-bone marrow hematopoietic sites in different organs.

CONCLUSION

The current research recommended consequent screening of mononuclear cells in peripheral blood followed by those of bone marrow for detection of HCV-RNA genomic materials by cellular-PCR to diagnose OCI in IgG-seropositive and seronegative non-viremic patients. Normal bone marrow function is one of the major requirements for HCV-RNA persistent viremia.

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