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Seronegative And Occult Hepatitis C Virus Infection in Patients with Acute and Chronic Myeloid Leukemia

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ABSTRACT

Background: Around the world, Hepatitis C (HCV) is the primary cause of liver disease. HCV infection in absence of a positive HCV test (Occult hepatitis C) is a significant clinical and epidemiological issue, although the pathogenic mechanisms behind this illness are not well comprehended.

Objective: This study aimed at investigation of seronegative and occult hepatitis C virus among patient with acute and chronic myeloid leukemia.

Patients and methods: 85 leukemic patients were studied in the current cross-sectional trial. In accordance with the kind of myeloid leukaemia (ML), patients were divided into 72 patients with acute ML (AML group) and 13 patients with chronic ML (CML group). And according to the presence of occult HCV infections, they were divided into 12 patients (14.1%) with negative serum HCV Abs and positive serum HCV RNA (seronegative), while the other 73 (85.9%) were free of HCV RNA in their bloodstreams.

Results: Patients with occult HCV infections had significantly higher monocytic count and significantly lower platelet count. Also, they had significantly lower total plasma protein and serum creatinine levels compared to those in the seronegative group. Patients with CML had either normal platelet count or even thrombocytosis, while those with AML showed thrombocytopenia with a statistical significant difference. Patients with AML had significantly higher AST.

Conclusions: Seronegative and occult HCV are significant clinical problems in leukemic patients and needed to be more and more evaluated.

Keywords: Seronegative HCV, Occult HCV, Myeloid leukemia.

INTRODUCTION

One of the primary causes of liver illness in the world is hepatitis C virus (HCV), which causes chronic viral hepatitis that frequently develops to liver necrosis and cancer of the hepatocytes [(1)]. Egypt has the world's highest rate of HCV infection (15 percent . Some communities around the Nile in Egypt have prevalence rates in excess of twenty two percent [(2)].

Despite the existence of HCV RNA in some patients, anti-HCV antibodies (Abs) are not present in these individuals. This is known as "seronegative" HCV infection, and it may be associated in immunocompromised cases of hemodialysis, onco-hematological illness, as well as HIV [(3)].

If hepatitis C (HCV) RNA is found in the liver and blood mononuclear cells (PBMCs) but not in serum, it is known as "occult HCV infection (OHC)" It can occur in anti-HCV positive patients with normal serum levels of liver enzymes, as well as those who have persistently elevated liver enzymes of unknown origin [(4)].

Patients with OCI may not be able to detect HCV RNA in their blood because of a mystery. Conventional molecular approaches may be incapable of detecting modest levels of circulating virus particles in patients with OCI. Another mystery is why these people fail to produce antibodies to the pathogen. Mutant HCV strains that generate antibodies but are not identified by currently available serological testing could be the source of concealed infection [(5)].

Infection with hepatitis C is transmitted by injection. Infection with the human hepatitis C virus (HCV) can occur in people who often come into contact with blood and blood products. Everyone from healthcare workers to those with haematological diseases to those on dialysis is at risk for the HCV virus [(6)]. In different regions of the world, studies on the link between seronegative and OCI HCV infections and haematological diseases have produced conflicting results [(7)].

We aimed in this work at investigation of seronegative and occult hepatitis C virus among patient with acute and chronic myeloid leukemia.

MATERIAL AND METHODS

This cross-sectional, comparative study was conducted on Eighty five subjects including both male and female at Oncology and Hematology Unit in Clinical Pathology Department, Zagazig University Hospitals in the duration between January 2019 and January 2020.

Inclusion criteria: Negative anti-HCV Abs test in all individuals with myeloid leukaemia, either AML or CML. Who were under the age of 18. Hepatitis B surface antigen (HBVs Ag) and anti-HIV Abs were included.

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Exclusion criteria: Those who were seropositive for anti-HCV Abs, HBVs Ag and anti-HIV Abs were excluded from this study.

According to type of myeloid leukemia (ML), patients were divided into 72 patients with acute ML (AML group) and 13 patients with chronic ML (CML group). And, according to the presence of occult HCV infections, they were divided into 12 patients (14.1%) with negative serum HCV Abs and positive serum HCV RNA (seronegative), while the other 73 (85.9%) were negative for HCV RNA in their bloodstream. After examination of HCV RNA in PBMNCs in the 73 cases; 18 patients which represent (21.2%) from total examined patients with negative serum HCV Abs and RNA HCV and positive HCV RNA in peripheral blood mononuclear cells "PBMCs" (occult c), while the other 55, which represent 64.7% from total examined patients were negative for HCV in serum and PBMNCs. After full medical history was taken, all subjects underwent a thorough physical examination.

Laboratory investigations:

Complete blood count, peripheral blood examination, erythrocyte sedimentation rate, and coagulation profile are all included in this procedure. PBMCs isolation was done for all patients and RNA examination were done in PBMCs and serum.

Specific examination:

1- Isolation of peripheral blood mononuclear cells (PBMCS):

Standard Ficoll-Paque techniques were used to separate PBMCs from whole blood (GE Healthcare, Uppsala, Sweden) according to the following steps:

- Venous blood samples were collected in a glass tube with EDTA and shaken perfectly.
- Centrifuge were done until plasma separation occurs and 400 µl of plasma were collected and preserved.
- The rest of the sample was diluted with PBs "phosphate buffer saline" then we layered sample on 3 ml Ficol and then centrifuged for 30 min at 500 rpm until the buffy coat was formed in the falcon tube.
- The buffy coat was collected in a new falcon tube and washed by PBs then centrifuged again at 4800 rpm for 10 min.
- We took the bellet of the buffy coat in ependorf and centrifuged again at12000 rpm for 2 min.
- At last we collect the bellet of the buffy coat and add 200 µl of plasma.

2- Extraction of RNA:

Pure Link RNA Mini Kit was used to extract the RNA (Table 1), (Invitrogen, USA, catalog number 12183018A) according to manufacturer protocol..

A. Preparations :

- **Preparing Wash Buffer II with ethanol :** Sixty mL of absolute ethanol were added directly to the wash buffer II bottle before using it for the first time .
- **Preparing Lysis Buffer with 2-Mercaptoethanol :** Each purification operation required a new batch of Lysis Buffer containing 1% 2-mercaptoethanol. Each 1 mL Lysis Buffer contained 10L of 2-mercaptoethanol, which was dissolved in the lysis buffer.
- **Centrifuge was adjusted at 12,000 × g.**

B. Steps :

- 200 µL of whole blood sample were placed into a 1.5 mL RNase–free microcentrifuge tube. Then 200 µLof previously prepared Lysis Buffer were added. The blood cells were disrupted by vortexing, For 2 minutes, the lysate was centrifuged . RNase-free micro centrifuge tubes were used to centrifuge the supernatant. The microcentrifuge tube was filled with 200 L of pure ethanol. Vortexing was used to ensure that the mixture was properly dispersed. Centrifuged at 15 rpm for 15 seconds, the sample was transferred to the spin cartridge. The sewage was drained and disposed of.
- A wash 700 µlitres For 15 seconds, It was spun in the spin cartridge. It was discarded and the spin cartridge was reinserted in a fresh Collection Tube. The spin cartridge was loaded with 500 µlitres of Wash Buffer II, and the mixture was centrifuged for 15 seconds. The sewage was drained and disposed of, and the spin cartridge was reinserted in the same collection tube. The process was then repeated a second time.
- The membrane with bound RNA was dried by centrifugation for one minute in a spin cartridge. After discarding the collection tube, a recovery tube with the spin cartridge was used. The spin cartridge was filled with 30 µL RNase-free water and incubated at room temperature for 1 minute. A centrifuge was used for 2 minutes to extract RNA from the spin cartridge and recovery tube.

C. Measures to avoid contamination :

- Preliminary disinfection and sterilization with UV rays were used to sterilise the laminar flow hood where the work was carried out.
- The work area for the amplification reactions was isolated from the rest of the lab.
- For protection against RNase contamination, plastic gloves were always used.
- When working with the given reagent, only RNase-free disposable filtered plastic pipette tips were utilized.

3- RNA quantitation and quality assessment:

The concentration of RNA was measured in each sample using Quantus fluorometer (Promega, USA). The input amount of total RNA in each sample was adjusted to 50 ng/µL. 10 µL were used for the next step..

4- PCR Analysis:

This was accomplished with the help of the Thermo Scientific GeneJET Viral DNA and RNA Purification Kit. 1300 µl of Reconstitution solution add to the tube with lyophilized IC (internal control). Then, incubate at room temperature for not less 10 min and periodically mix thoroughly by vortex. 800 µl of Reconstitution solution were add to the tube with lyophilized Standards. Then, incubate at room temperature for not less 10 min. and periodically mix thoroughly by vortex. The amplification mix was prepared by mixing necessary amounts of reagents. 10 ul of resulting amplification mix was added to prepared tubes. By using an aerosol filter to all tubes, 15 ul of: (a) In negative control sample tube – negative control extraction sample. (b) In tubes of analysed samples – analysed RNA samples. (c) In standard sample tubes – dissolved standard samples (from reagent kit). All tubes were closed centrifuged for 3-5 seconds at 1500-3000 rpm on a microcentrifuge. The tubes was transferred into PCR instrument for performing of PCR with real time detection.

Ethical approval:

Research Ethics Council at Zagazig University accepted the study after receiving written informed concents from all patients. It was conducted in accordance with the Declaration of Helsinki, the Code of Ethics of the World Medical Association, which governs all human research.

Statistical analysis

SPSS 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA) and MedCalc 13 for Windows were used to gather, tabulate, and statistically analyse all of the data (MedCalc Software BVBA, Ostend, Belgium). The mean standard deviation, mean, and range were used to represent continuous data, while the percentage was used to describe categorical data. To compare two groups with normally distributed data, we utilized an independent student t-test. The Chi-square (χ^2) test was used to compare the percentage of categorical variables. The Pearson correlation coefficient was used to calculate correlations between variables. P 0.05 was deemed statistically significant, whereas P 0.01 was deemed extremely significant.

RESULTS

Men made up 52.9% of the study's participants, with an average age of 37.87 ± 10.38 years. Serum HCV RNA (positive PCR) was reported in 55 (64.7%) of our cases, while the other 30 (35.3%) were negative for serum HCV RNA. And, according to the presence of occult HCV infections, they were divided into 12 patients (14.1%) with negative serum HCV Abs and positive serum HCV RNA (seronegative), while the other 73 (85.9%) were negative for serum HCV RNA.

After examination of HCV RNA in PBMNCs in the 73 cases; 18 patients which represent (21.2%) from total examined patients with negative serum HCV Abs and RNA HCV and positive HCV RNA in peripheral blood mononuclear cells "PBMCs" (occult c) , while the other 55, which represent (64.7%) from total examined patients were negative for HCV in serum and PBMNCs .

In terms of clinical data, treatment response, and type of leukaemia, there was no statistically significant difference between OCI and seronegative patients. Comparing the two groups' platelet, MCV, and MCH levels revealed statistically significant differences. For liver and renal function tests, there was no statistically significant difference between the two groups (Tables 1 and 2).

AML was reported in 72 (84.7%) of our cases and CML was reported in 13(15.3%). There was no statistically significant variation in the age and sex distribution of the AML and CML groups. Also, there was a statistically significant difference between both groups regarding platelet and mean corpuscular hemoglobin (MCH) with less platelets count in AML patients. Patients with AML and CML had significantly different AST levels, which was statistically significant.

Table (1): Comparing laboratory hematological results between patients with AML and patients with CML

Variable	AML Mean± SD	CML Mean± SD	p value
Total WBCS x l0^3 / uL	**21.042±2.804**	**22.6±3.64**	**0.208**
Neutrophil x10^3 / uL	**4.671±0.623**	**5.03±0.81**	**0.05**
Monocyte x 10^3/ uL	**9.343±1.245**	**10.06±1.62**	**0.259**
Lymphocyte x 10^3/ uL	**6.94±0.926**	**7.48±1.2**	**0.826**
Hb g / d L	**9.288±1.963**	**10.061±1.744**	**0.189**
MCV FL	**81.166±4.385**	**79.107±3.834**	**0.117**
MCH pg	**28.741±2.152**	**27.476±1.271**	**0.044***
PLT x 10^3/ u L	**58.541±11.095**	**131.384±8.987**	**0.006***
ESR	**59.111±3.366**	**72.153±8.618**	**0.06**
INR	**1.403±0.407**	**1.700±0.001**	**0.129**

Table (2): Comparing liver and kidney functions between patients with AML and patients with CML

Variable	AML Mean± SD	CML Mean± SD	p value
Direct bilirubin mg / dl	**0.508±0.173**	**0.66±0.18**	**0.779**
Total bilirubin mg /dl	**0.871±0.17**	**0.93±0.2**	**0.337**
Total protein g /dl	**6.793±0.924**	**6.754±0.697**	**0.887**
Albumin g/dl	**3.736±0.712**	**3.441±0.625**	**0.166**
ALT u/ L	**31.409±4.850**	**15.00±2.815**	**0.065**
AST u/L	**22.238±3.530**	**14.461±3.104**	**0.035***
Creatinine mg/ dl	**0.658±0.155**	**0.836±0.151**	**0.175**
Urea mg /dl	**13.409±2.739**	**17.784±2.037**	**0.092**

Table (3): Comparing hematological results between seronegative and OCI patients

Variable	Seronegative Mean± SD	Occult HCV Mean± SD	p value
Total WBCS x $l0^3$ / uL	**22.56 ±4.28**	**20.51 ± 1.82**	**0.080**
Neutrophil x10^3 / uL	**5.01 ± 0.95**	**4.55 ± 0.40**	**0.084**
Monocyte x 10^3/ uL	**10.02 ± 1.90**	**9.11 ± 0.81**	**0.080**
Lymphocyte x 10^3/ uL	**7.44 ± 1.41**	**6.77 ± 0.60**	**0.131**
Hb g / d L	**9.19 ± 1.85**	**9.19 ± 1.02**	**0.082**
MCV FL	**73.11 ± 5.32**	**79.04 ± 5.0**	**0.004***
MCH pg	**26.03 ± 2.18**	**28.04 ± 2.20**	**0.013***
PLT x 10^3/ u L	**156.67 ± 14.81**	**53.94 ± 7.24**	**<0.001***
ESR	**52.83 ± 6.38**	**66.06 ± 3.15**	**0.168**

Hb: Hemoglobin; **WBC:** White Blood Cells; **PLT:** platelets; **ESR:** erythrocyte sedimentation rate.

Table (4): Comparing liver and kidney function tests between seronegative and occult HCV patients

Variable	Seronegative Mean± SD	Occult HCV Mean± SD	p value
Direct bilirubin mg / dl	**0.82 ± 0.14**	**0.91 ± 0.12**	**0.560**
Total bilirubin mg /dl	**0.48 ± 0.15**	**0.65± 0.15**	**0.279**
Total protein g /dl	**6.0 ± 0.91**	**6.44 ± 0.8**	**0.173**
Albumin g/dl	**3.27 ± 0.94**	**3.48 ±0.620**	**0.465**
ALT u/ L	**22.73 ±1.44**	**25.73 ± 2.93**	**0.747**
AST u/L	**24.30 ± 5.84**	**18.23 ±1.39**	**0.231**
Creatinine mg/ dl	**0.67 ± 0.13**	**0.54 ± 0.16**	**0.077**
Urea mg /dl	**14.28 ± 1.64**	**11.29 ± 2.62**	**0.298**

ALT: Alanine Transaminase; **AST:** Aspartate Aminotransferase.

Table (5): correlation between total WBCs, liver enzymes and other laboratory results in AML cases

Items		Total WBC x l0^3 / uL	ALT u/ L	AST u/ L
Total wbcs x l0^3 / uL	r	----------	-.048	.056
	P value		.686	.641
ALT u/ L	r	-.048	----------	.730**
	P value	.686		.000
AST u/L	r	.056	.730**	----------
	P value	.641	.000	
Hb g / d L	r	.275*	.496**	.446**
	P value	.019	.000	.000
MCV FL	r	.034	.352**	.262*
	P value	.778	.002	.026
MCH pg	r	.063	.350**	.279*
	P value	.599	.003	.018
PLT x 10 ^ 3 / u L	r	.529**	.027	.050
	P value	.000	.822	.680
Bil T mg /dl	r	-.004	.169	.150
	P value	.975	.156	.207
Bil D mg / d L	r	.024	.155	.140
	P value	.843	.193	.242
Total protein g / d L	r	.140	-.092	.011
	P value	.241	.442	.924
Albumin g/dl	r	.341**	.114	.161
	P value	.003	.341	.175
Creatinine mg/ dL	r	-.116	-.080	-.035
	P value	.333	.504	.767
Urea mg /dL	r	-.206	-.083	-.149
	P value	.083	.487	.212
ESR	r	-.032	-.313**	-.188
	P value	.792	.007	.113

Table (6): Correlation between total WBCs, liver enzymes and other laboratory results in CML cases.

Items		Total WBC x l0^3 / uL	ALT u/ L	AST u/ L
Total wbcs x l0^3 / uL	r	----------	.199	.910**
	P value		.515	.000
ALT u/ L	r	.199	----------	.010
	P value	.515		.974
AST u/L	r	.910**	.010	----------
	P value	.000	.974	
Hb g / d L	r	-.305	.408	-.382
	P value	.311	.167	.198
MCV FL	r	.821**	.604*	.681*
	P value	.001	.029	.010
MCH pg	r	.696**	.544	.700**
	P value	.008	.055	.008
PLT x 10 ^ 3 / u L	r	.799**	-.169	.753**
	P value	.001	.580	.003
Bil T mg /dl	r	.445	-.307	.596*
	P value	.127	.308	.031
Bil D mg / d L	r	.807**	-.252	.901**
	P value	.001	.406	.000
Total protein g / d L	r	-.486	.145	-.550
	P value	.092	.638	.052
Albumin g/dl	r	-.777**	.154	-.949**
	P value	.002	.616	.000
Creatinine mg/ dL	r	.467	.338	.478
	P value	.108	.259	.098
Urea mg /dL	r	.927**	.270	.879**
	P value	.000	.373	.000
ESR	r	-.148	.137	-.053
	P value	.630	.655	.864

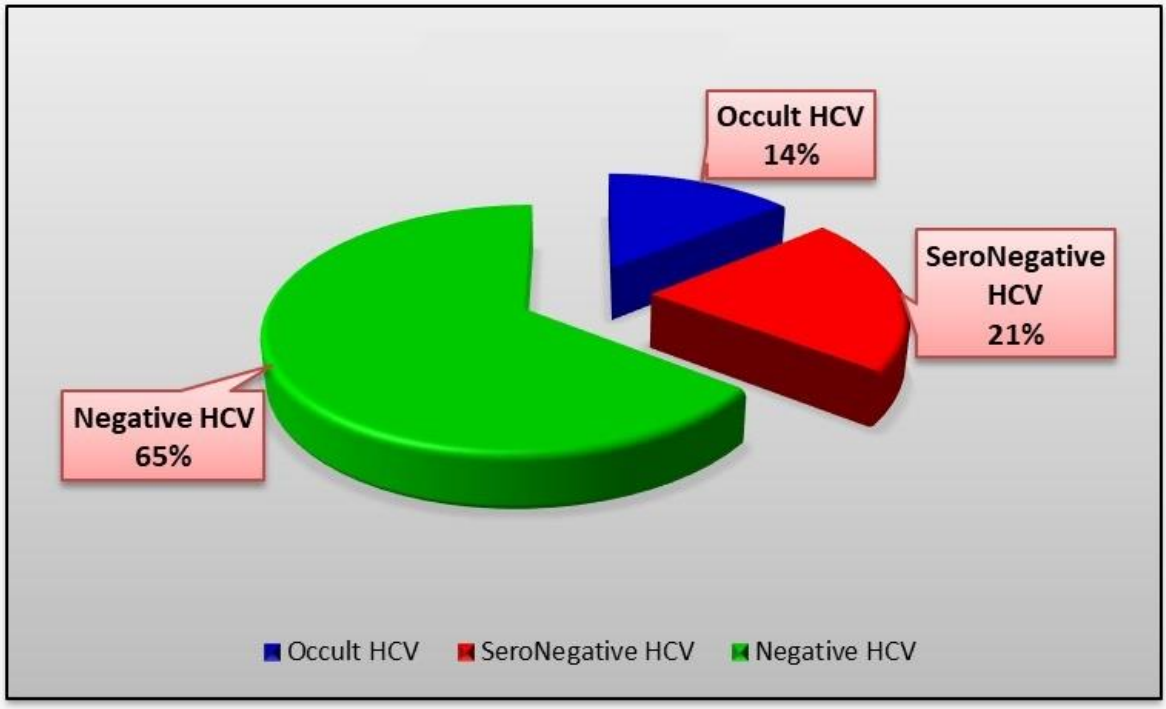

Figure (1): PCR results of the studied patients

DISCUSSION

Hepatitis C virus is the cause of hepatitis C, an infectious liver disease. For many years, hepatitis might go unnoticed, but if chronic infection is established, scarring of the liver (fibrosis) and advanced scarring (cirrhosis) can develop [8].

Patients with acute myeloid leukaemia (AML) made up 72.7% of the total, while patients with chronic myeloid leukaemia (CML) made up 15.3% of the patient population. In agreement with our findings are **Bayraktar and Goodman**[9] and **Redaelli *et al.*** [10] who reported that About 7–20% of all instances of leukaemia are CML, a myeloproliferative condition with an occurrence rate of 2 occurrences per 100,000 people.

We compared between patients with AML and those with CML regarding complete blood counting parameters and platelet count and mean corpuscle haemoglobin "MCH" that were statistically significantly different between the two groups, with p-values of 0.0444 and 0.06 correspondingly. Patients with CML was found to have either normal platelet count or even thrombocytosis (131.38 ± 68.99) while those with AML were found to have thrombocytopenia (58.541 ± 111.095). Other CBC parameters and ESR and INR were not significantly different between the two groups. **Kumar *et al.*** [11] found that roughly 68.9 percent of CML patients had platelet counts in the range of 100–450 10^3/dL, which is consistent with these findings. Thrombocytosis (higher than 450x 10^3/dL) was found in 24.4% of the patients. The fact that thrombocytopenia was found in all individuals with AML was reported by **Arepally** [11]. Nineteen (38%) of patients showed severe degree of thrombocytopenia (platelet count < 50×109/l) and 19 (38%) had moderate to mild degree (50-100×109/l) and 12 (24%) patients with lower limit of normal platelet count that is <150×109/l.

Regarding liver and kidney functions, our results showed that patients with AML had significantly higher AST with p-value: 0.035. While, there was no significant difference was detected as regards other parameters.

All selected patients were negative for presence of anti-HCV antibodies and after doing PCR for presence of HCV RNA in serum and/or PBMCs and our results showed that serum HCV RNA (positive PCR) was reported in 55 (**64.7%**) of our cases, while the other 30 (**35.3%**) were negative for serum HCV RNA. After examination of HCV RNA in PBMNCs we found that 73 (**85.9%**) cases hsd positive HCV RNA in PMNCs while the other 12 (**14.1%**) were negative. Occult HCV was detected in 18 (**21.2%**) from all cases, while the other 55 (**64.7%**) were seronegative from all cases . Anti-HCV negative patients with either benign or malignant haematological disorders (group I) and 20 age- and gender-matched apparently healthy subjects were studied by **Helaly *et al.*** [1] and found that 60 (66.7 percent) of the 90 subjects had seronegative HCV infection, while 18 (20 percent) had occult HCV infection.

We compared between seronegative patients and those with occult HCV infection as regards basic demographic characteristics and our results showed that there was no statistical significant difference between both groups as regards age and sex distribution with p-value: 0.148 and 0.17 respectively. Against our finding regarding age is **Helaly *et al.*** [1] who reported that there was a statistical significant difference between patient with occult HCV and seronegative patients. While

regarding sex distribution they were in agree with our findings.

Also, we compared between patients groups regarding CBC parameters, ESR and INR and our results showed that patients with occult HCV infections had significantly higher MCV and MCH and significantly lower platelet count, while, there was no statistical significant difference between both groups as regards other variables.

With regard to testing for liver and renal function we found no statistically significant difference between the two groups. Only 8.57 percent of individuals with OCI exhibited increased ALT readings in a study by **Martnez-Rodrguez *et al.*** [(12)] OCI may have a slower progression to cirrhosis than CHC, therefore those people may be at an early stage of infection. Against our findings regarding ALT and AST were **Helaly *et al.*** [(1)] who reported that seronegative and occult HCV infection groups had statistically significant differences in AST activity, but not ALT activity, while seronegative HCV infection or OCI and negative HCV groups had statistically significant differences in ALT and AST activity.

Even in people with normal liver enzyme levels who are anti-HCV positive, occult HCV infection can occur; the cause of occult HCV infection remains a mystery. As a result, measuring ALT and AST levels is not a critical step in determining whether a patient has OCI [(2)].

CONCLUSION

From the results of this study, we concluded that seronegative and occult HCV are significant clinical problems in leukemic patients and needed to be more and more evaluated.

RECOMMENDATIONS

Further studies on larger population are needed to clarify the real extent of these clinical problems. Liver functions test alone are not good indicators of presence of occult HCV infections. For diagnostic purposes, more comprehensive molecular tests should be used in conjunction with routine assessments of liver function, particularly in patients who require frequent blood transfusions or who are taking immunosuppressive medications.

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