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Molecular Detection of Cytomegalovirus in A Sample of Iraqi Patients with Acute Leukemia and Stem Cell Transplantation

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Abstract

Background Acute leukemia and hematopoietic stem cell transplantation are risk factors for opportunistic infection and reactivation of many latent infection like cytomegalovirus.

Objective Detection and quantification of cytomegalovirus viremia in patients with acute leukemia after induction chemotherapy and post allogeneic stem cell transplantation patients.

Methods A prospective study enrolled 61 patients with acute leukemia. Forty-eight of them evaluated while induction chemotherapy (group I), while the other 13 within 1-year post bone marrow transplantation (BMT) (group II). In addition, 30 apparently healthy individuals were recruited as (control group), blood samples were collected from all groups. Viral DNA was extracted from 1 ml plasma samples, and then, cytomegalovirus DNA was detected and quantitatively assessed by Taqman quantitative real-time PCR.

Results Twelve (25%) out of 48 patients in group I, 2 (15.4%) out of the 13 patients in group II, and 2 (6.7%) out of 30 in the control group had positive cytomegalovirus viremia. The mean cytomegalovirus viremia was 5.192x102, 2.71x102 and 1.60x102 copies/ml for group I, group II and controls respectively, p=0.056.

Conclusion There is a relatively high prevalence of cytomegalovirus viremia in Iraqi patients with acute leukemia after chemotherapy and post BMT. Real-time PCR assay is helpful for early diagnosis of cytomegalovirus viremia in leukemic patients and used to monitor post BMT patients at risk for cytomegalovirus disease.

Keywords HCMV, acute leukemia, stem cell transplantation, real-time PCR

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List of abbreviations: ALL = acute lymphoblastic leukemia, AML = acute myeloblastic leukemia, CBC = Complete blood count, DM = diabetes mellitus, HBV =hepatitis B virus, HCMV = human cytomegalovirus, HCV = hepatitis C virus, HT = hypertension, QRT-PCR = Quantitative real time polymerase chain reaction, SCT = stem cell transplantation

Introduction

Acute leukemia is aggressive disease in which malignant transformation occurs in hemopoietic stem cell or early progenitors [(1)]. Acute leukemia has two broad classifications: acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML) [(2)]. In acute leukemia, normal hematopoiesis is replaced by immature cells and deregulated proliferation of leukocytes [(3)]. Chemotherapy and stem cell transplantation (SCT) are the established therapeutic options for these patients. These methods cause an increased

risk of infections [4]. Patients undergone SCT are more susceptible because their immune system is depressed by immunosuppressive therapy and the immune reconstitution is not fully developed. This can lead to severe infection [5].

Cytomegalovirus (HCMV) belongs to the human herpes viruses, beta herpes viruses. It is a major pathogen causing significant mortality in immunocompromised hosts [6,7]. It has double-stranded DNA (dsDNA) genome, which is longer than all other human herpes viruses [8]. HCMV can infect a wide range of cells within its host, including various hematopoietic cell types and connective tissue and parenchymal cells of any organ [9]. HCMV seropositivity is common in the general population, with a prevalence ranging from 30-97%. After the primary infection, HCMV establishes a life-long latency in various organs [10-12]. HCMV has a wide spectrum of clinical presentation. It can present generally as asymptomatic and persistent infections in healthy persons. However, it can also lead to serious disorders among transplant recipients, immunodeficient patients, patients on immunosuppressive treatment [13], and patients with hematological malignancies [14].

Most of the recent studies showed high incidence of HCMV infection in leukemia patients [15,16]. In addition, HCMV infection is a major infectious complication after allogeneic hematopoietic cell transplantation (allo-HSCT) [17-19]. Early HCMV reactivation remains associated with increased transplant-related mortality [19]. A previous study in Iraq revealed a 28% prevalence of HCMV in ALL patients. However, the best of our Knowledge, there is no similar study regarding HCMV in overall acute leukemia [20].

This study aimed to detection of HCMV in patients with acute leukemia after chemotherapy (induction) courses and post allogeneic stem cell transplantation patients within the first year, and to determine copy number of HCMV in these groups and compare with apparently healthily individuals.

Methods

Study population

A prospective study conducted from 1st of December 2016 to 1st of June 2017. Sixty-one (61) patients with acute leukemia were enrolled in this study. Forty eight (78.7%) of them had received an induction course of chemotherapy within one month of diagnosis as group I. Those are comprised as 18/48 (37.50%) patients with ALL and 30/48 (62.50%) with AML. They collected from Hematology Ward at Al-Imamein Al-Kadhimein Medical City and Baghdad Teaching Hospital, Medical Complex. The rest thirteen (21.3%) acute leukemia patients (5 ALL and 8 AML) had assessed after bone marrow transplantation within the first year of diagnosis as group II and collected from the Bone Marrow Transplantation Center in the Medical Complex, Private Nursing House. Thirty apparently healthy individuals from volunteers and donors in the blood bank who served as control group. A consent letter was obtained from all patients and controls enrolled in the study. This study approved by the Institutional Review Board of the College of Medicine, Al-Nahrain University. Clinical and laboratory data were obtained from all patients from records and controls by direct interview. Blood sample were collected in EDTA tube form study groups and 1 ml of plasma was separated and preserved in deep freeze for viral DNA extraction.

Viral DNA extraction

For viral DNA extraction from the plasma samples; Geneius™ Viral Nucleic Acid Extraction Kit III (Geneaid, Taiwan) was used. One ml plasma was used in viral DNA extraction, according to the manufacturer protocol.

Real Time PCR for measuring HCMV viremia

For the quantitative detection of HCMV; HCMV dtec-qPCR Test F-100 Kit (Genetic PCR Solutions TM, Spain) a Real-Time test, which is based on the principle of the so-called - "TaqMan" probe was utilized. Fifteen µl of Master Mix were added into PCR tubes, and 5

µl of the (sample DNA, positive or negative controls, or standards) were added to the master mix. The final reaction volume was 20 µl. All components were kept at room temperature during the PCR preparation. Real time PCR instrument used in this work was STRATAGENE MxPro QPCR (Agilent Technologies, USA). The thermal protocol for HCMV dtec-qPCR kit is composed of a two hold steps, and one amplification cycle. The real time data is collected at the second step of the amplification cycle as demonstrated in table (1).

At the end of the thermal protocol, the Real Time PCR (MxPro QPCR) instrument software automatically calculates the baseline cycles and the threshold. The standard curve is plotted using the data obtained from the defined standards, with the (Y) axis is the Ct-Threshold Cycle, and the (X) axis is the viral DNA copy number. According to the manufacturer instructions, HCMV DNA copies were calculated depending on to the following formula:

$$\text{copy/ml} = \frac{\text{SC x EV}}{\text{IV}}$$

SC = Sample Concentration (copy/µL)
EV = Elution Volume
IV= Isolation Volume (ml)

Table 1. HCMV real time PCR amplification profile

Step		Time	Temperature
Activation 1		15 min	95 °C
40 cycles	**Denaturation**	15 sec	95 °C
	Hybridization/Extension and data collection 2	60 sec	60 °C

*1- step one; **2- step two

Statistical analysis

Microsoft excel 2016 and SPSS (statistical package for social sciences) version 23 was used for statistical analysis. Most of the data were numerical so presented as mean ± standard deviation, and comparison between means of study groups was done by using independent student t-test. Categorical data were presented as frequency and percentage; fisher exact test, and chi-square test and Mann Whitney test were used for comparison between frequencies of study groups. P value less than 0.05 was considered as significant.

According to test of normality (Shapiro-wilk) most of data, were not normally distributed for this we used the Mann Whitney test, WBC < 0.001, Neutrophil < 0.001, lymphocyte < 0.001, Hb < 0.057, platelets < 0.001.

Results

The ratio of males was the predominant, 56.3% (27/48), 76.9% (10/13) and 56.7% (17/30) in group I, II and control respectively, while the mean age was 37.27 ± 15.66, (range of 14-70 years); 29.77 ± 14.45 (range of 12-56 years) and 30.87± 10.58, (range of 14-53 years) respectively. Statistically, there was no significant difference (p=0.076) between the mean age of the patients and control indicating that they were of a comparable age.

Quantitative real time PCR (QRT-PCR) run gave positive viremia in (25.0%) 12 out of 48 in group I and (15.4%) 2 out of 13 in group II as well as (6.7%) 2 out of 30 in control, however, these results were statistically not significant (p=0.056) in the mean of the copy numbers in all groups. Table (2) shows that the mean copy number in group I was (519.17 ± 236.44), group II was (271.0 ± 24.04) and the mean of copy number in control was (160.0 ± 4.24).

Table 2. Comparison of HCMV copy number in different study groups by ANOVA

Parameter	Group I copy/ml	Group II copy/ml	Control copy/ml	P value
HCMV copy no.	0.379×10^3 0.409×10^3 0.384×10^3 0.511×10^3 0.518×10^3 0.670×10^3	0.288×10^3	0.163×10^3	0.056
	0.405×10^3 0.207×10^3 0.881×10^3 0.427×10^3 1.045×10^3 0.394×10^3	0.254×10^3	0.157×10^3	
Mean	519.17	271.0	160.0	
SD	236.44	24.04	4.24	
Range	$(0.207\text{-}1.045)\times10^3$	$(0.254\text{-}0.288)\times10^3$	$(0.157\text{-}0.163)\times10^3$	

*Cut-off level of this method was 0.150×103 copies/ml. HCMV viremia was defined by positive HCMV -specific RT-PCR in plasma [21]

According to type of leukemia in group I and II, 2 (8.7%) patients with ALL and 12 (31.6%) patients with AML positive for HCMV. There was a significant association between HCMV viremia and type of leukemia (p value = 0.012) as in table (3).

Table 3. Comparison of the results of HCMV viremia according to type of acute leukemia in group I and II

HCMV	ALL (group I + II) N=23 No. (%)	AML (group I + II) N=38 No. (%)	P value
Positive	2 (8.7)	12(31.6)	0.012
negative	21 (91.3)	26 (68.4)	

Relationship between the HCMV viremia and the demographic data

Data of this study revealed that there was no significant difference in the age of the three groups and HCMV viremia. Group I (p value = 0.582), age group (20-39) was the most frequently reported in all groups. Regarding gender, males were predominant in group I. HCMV was positively expressed more in male where 7 out of 12 patients. In group II patients HCMV viremia was detected in 2 male patients, whereas two females in control group. No statistically significant difference was found among sex in the 3 groups.

Relationship between the HCMV viremia and history of blood transfusion and co-infection

There was no significant association between HCMV positive viremia and blood transfusion (p value = 0.601, 0.656) in group I and group II respectively. Similar results were reported in relation to co-infection with HBV as there is no statistical significance in both groups (p value =

0.08, 0.512). Only one patient was positive for HCMV and HBV together.

Relationship between the HCMV and the hematological parameters

Statistically significant difference according to hematological parameters between the HCMV positive and HCMV negative patients (group I and group II) was found as shown in table (4).

Table 4. Relationship between HCMV positivity group I and group II with hematological parameters

Parameter		HCMV Negative N=47	HCMV Positive N=14	P value
WBC (*10^3/µl)	Mean	10.46	2.44	0.004
	SD	19.11	1.59	
	Median	4.80	2.42	
	Range	0.2-86.43	0.26-4.7	
Neutrophils (*10^3/µl)	Mean	4.26	0.90	0.01
	SD	6.56	0.70	
	Median	1.55	0.71	
	Range	0.2-30.57	0.01-2.06	
Lymphocytes (*10^3/µl)	Mean	3.71	0.90	0.015
	SD	8.10	0.44	
	Median	1.42	1.05	
	Range	0.01-40.39	0.24-1.4	
Hemoglobin (g/dl)	Mean	9.76	7.94	0.045
	SD	3.13	1.43	
	Median	9.30	7.75	
	Range	4.2-16.4	4.7-10.6	
Platelets (*10^3/µl)	Mean	124.36	64.64	0.03
	SD	95.63	73.10	
	Median	96.00	36.50	
	Range	7-355	12-260	

*Leukopenia (WBC count < 4.0×103/µl) [22].
*Neutropenia (neutrophil count <1.5 ×103/µl) [23].
*Lymphopenia (Lymphocytes < 1.0×103/µl) [24].
*Anemia (hemoglobin < 12 g/dl) [25].
*Thrombocytopenia (platelets<150×103/µl) [26].

Discussion

This study revealed that 12 out of 48 (25%) of acute leukemia patients were positive for HCMV viremia, distributed as 10 out of 30 (33.3%) of AML positive to HCMV, which is comparable to other studies such as Capria et al. 2010 [27], in which 35% (21/59) patients in complete remission after chemotherapy were HCMV positive. On the other hand, out of 18 ALL patients, only 2 (11.11%) were HCMV positive. This result is comparable to that of Han et al. 2007 [28] who reported HCMV viremia in 11.1% of ALL cases and Jain et al. 2016 [29] who reported it to be in 10% of children with ALL by PCR.

Out of those patients who were studied after bone marrow transplantation only two out of 13 (15.4%) were HCMV positive, which disagreed with other studies, accomplished by Guenounou et al. 2016 [30] who reported it to be 49/136 (36%) and Poiré et al. 2017 [31] who reported that 84/125 (68.3%). Important

explanation is patient under prophylaxis drug, the serostatus for HCMV of donors and recipients before transplantation, restricted accessibility for all patients and may be due to the small number of cases in the present study compared to previous studies. In the control group; only 2 out of 30 (6.66%) was positive for HCMV, this result is within range in comparison to other studies included study in Ouagadougou, Burkina Faso which was 5.1% by Traore et al. 2016 [32], and HCMV showed 10% seropositive in donors of blood bank of Mosul city by Al-Dabbagh et al. 2011 [33].

HCMV viremia and type of leukemia

The results of this study showed a significant association between HCMV viremia and type of acute leukemia; (P = 0.012). 12 AML and 2 ALL in acute leukemia (group I) and PBMT (group II); which is contrary to that of Dixon et al. 2017 [34] who showed that high HCMV positivity directly associated with ALL rather than AML. Also, another study by Han et al. 2007 [28] showed that HCMV expressed in 11.1% of ALL cases, while only 4% in AML. Many reasons standing behind this result including, the use of aggressive chemotherapy regimen in AML patients, or hospital acquired infection from para-medical personnel or form care giving career, in addition to another possible source for the primary infection which might be through blood transfusion where they transfused more regarding platelet and blood product transfusion. Another, possible explanation is that the AML samples are larger than ALL in this study and the different in number of chemotherapy courses between these studies.

Viremia and demographic data

There was no significant association between HCMV viremia with age and sex of the patients and control groups, a result which is supported by other reports from Loutfy et al. 2017 [35] and Loutfy et al. 2006 [36].

Relation HCMV viremia with blood transfusion and Co-infection

Regarding the correlation of HCMV positivity with the blood transfusion, it appeared that there was no significant relation. This result was consistent with that obtained by Pennap et al. 2016 [37] and Ojide et al. 2012 [38]. Another possible source for the primary infection might be that the blood donor was infected with active asymptomatic HCMV infection and may be viremia at the time of donation. A study reported that 3% of normal blood donors can be viremic during the time of donation and HCMV has been isolated from peripheral blood of healthy blood donors [39]. This study found no statistical significance association between HCMV viremia and HBV, compared to another study showing that the HCMV infection is common in chronic HBV patients, who can be regarded as patients at high risk for HCMV disease and those result was obtained by Bayram et al. 2009 [40].

HCMV viremia and hematological parameters

Regarding correlation between the hematological parameters and HCMV viremia in both groups, there was significant association between the mean WBCs count HCMV positive patients was lower than that in HCMV negative, (2.44 ± 1.59) x 10^3/µl vs. (10.46 ± 19.11) x 10^3/µl cells, respectively, p = 0.004, those findings were comparable with the results obtained by Loutfy et al. 2017 [36] and Jang et al. 2011 [41]. These studies clarified that the majority of patients positive to HCMV DNA was associated with leucopenia (p = 0.03, 0.012 respectively).

Intensive cytotoxic chemotherapy can cause severe and sometimes prolonged neutropenia, which may cause potentially fatal infection. Severe prolonged neutropenia is most likely to occur in the pre-engraftment phase of hematopoietic cell transplantation (HCT; particularly allogeneic) and in patients undergoing induction chemotherapy for acute leukemia. The mean neutrophil was higher in HCMV negative patients as compared to the HCMV positive patients, the differences in mean values of these parameters were statistically insignificant (p = 0.01) and those results in agreement with Loutfy et al. 2017 [35] and Jang et al. 2011 [41]. Who reported that HCMV viremia was mainly expressed in patients who had neutropenia.

In addition, the mean lymphocytes count in HCMV positive patients was lower than in HCMV negative patients, (0.90 ± 0.44) x 10^3/µl vs. (3.71 ± 8.10) x 10^3/µl, respectively, (p = 0.015), this result is comparable to that of Loutfy et al. 2017 [35], Jain et al. 2016 [29], which conformed that HCMV viremia is significantly associated with Lymphopenia.

The mean Hemoglobin level of HCMV positive (7.94 ± 1.43) g/dl was lower than (9.76 ± 3.13) g/dl of the HCMV negative, p = 0.045. Our result disagreed with Loutfy et al. 2017 [35]. Leukemic patients of different classifications are associated with anemia [42]; it may also be a result of patient's antineoplastic therapy or progressive disease and hemolysis. Acute infections with HCMV may lead to severe hematologic disorders. HCMV infection can also be associated with hemolytic anemia [43,44]. Platelets count was different also, and lower in HCMV viremic patients, in which it was significantly higher in HCMV negative patients, (64.64 ± 73.10) x 10^3/µl vs. (124.36 ± 95.63) x 10^3/µl cells, respectively, p = 0.03. This result agreed with Loutfy et al. 2017 [35]. It may also be early evidence of HCMV infection; Symptomatic thrombocytopenia may be early evidence of haematological disorders caused by HCMV infection [45].

This study concluded that patients with acute leukemia after chemotherapy or post BMT are at high risk of HCMV infection or reactivation.

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Authors' contribution

Al-Toban: Collection of specimens, DNA extraction, and real time-PCR run, writing of the manuscript and references. Dr. Al-Marsomy: Supervision. Dr. Al Tameemi: Consultant hematologist helped in selection of patients, providing of patients and writing of the manuscript. Dr. Al-Obaidi: helped in real time-PCR run. Dr. Mohammed: Consultant hematologist help in providing of patients. Dr. Al-Saeed: Consultant hematologist help in providing of patients. Dr. Al-Shemary: Consultant hematologist help in providing of patients.

Conflict of interest

The authors declare no conflict of interest.

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