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RESEARCH ARTICLE

THE ASSOCIATION OF HERPES VIRUSES TO LEUKEMIA IN SOME IRAQI PATIENTS

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ABSTRACT

Viruses, also known as oncoviruses, capable of transforming cells into a malignant phenotype. These viruses encode oncogenes that can be inserted into the mammalian genome causing transformation. This study was aimed to detect three types of herpes viruses HHV 4, HHV 6 and HHV 8 sequences in leukemia patients and healthy individuals to evaluate the role of these viruses in leukemia arise, and to determine the best way to detect the virus particles in human sera and blood. The present work were obtained four types of leukemia, Acute Lymphoid Leukemia (ALL), Acute Myeloid Leukemia (AML), Chronic Lymphoid Leukemia (CLL) and Chronic Myeloid Leukemia (CML). The Samples of blood were taken from 75 Iraqi patients newly diagnosed affected with one of the four types of leukemia aged between (2-63) years, and twenty-five healthy control subjects ages between (8-62) year were analysis by molecular genetic methods. During the period from August 2012 till May 2013. The viral DNA extraction from serum by QI Aamp Ultra Sens Virus Kit from Qiageen/Germany was found more suitable and sensitive to detect the present viruses than the genomic DNA extraction from whole blood, further amplified by Polymerase Chain Reaction (PCR). PCR products were analyzed by electrophoresis on 2% agarose gels. The present study detected EBV serologically in serum of patients and controls by the MONO mononucleosis rapid test. The results revealed the molecular way was more effective and sensitive that EBV detect in serology in 2.7% but with molecular methods detect in 19% of samples. These percentage of EBV divided on the four types of leukemia in different numbers, 12% in ALL, 3% in AML, 4% in CLL, where there was no positive samples to HHV-6 and HHV-8.

INTRODUCTION

Leukemia is cancer of the blood or bone marrow characterized by an abnormal increase of blood cells usually leukocytes (white blood cells). Decrease in the normal white blood cells (W.B.Cs) production will make the patient more susceptible to infections and bleeding due to not enough platelets available to produce homeostatic plugs (Hoffbrand *et al*., 2002). Some of neoplastic cells escape into blood will infiltrate into various other tissues producing general organ enlargement or less commonly tumor masses (Mackie *et al*., 1999). In Iraq, Leukemia ranks second among ten types of cancer in 2004 (Ministry of Health, 2002) while in 1989, it ranked seven among the ten types of cancer (Ministry of Health, 2010). Several factors have been identified as causes developing leukemia such as genetic and environment factors. Leukemia, like other cancers, results from somatic mutations that may occur spontaneously or due to family genetic history or as a result of exposure to carcinogenic substances (Ministry of Health, Iraqi Cancer Board, 1993). Some families have a genetic predisposition towards developing leukemia. Affected people may have a single gene or multiple genes in common.

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In some cases, families tend to develop the same kind of leukemia as other members; in other families, affected people may develop different forms of leukemia or related blood cancers (Rowley, 2001; Arzanian *et al*., 2006). Leukemia is also associated with a remarkable variety of acquired genetic abnormalities including chromosomal abnormalities and genes defects. Chromosomal translocations are the hallmark of many leukemias and frequently lead to the generation of chimeric fusion on coproteins that trigger malignant transformation (Aoun *et al*., 2007; Greaves, 1997; Gilliland *et al.,* 2004; Chad *et al.,* 2007). On the other hand, leukemia can arise from the exposure to various environment carcinogens. Viruses are among these carcinogens. Viruses are entities whose genomes are elements of nucleic acid that replicate inside living cells using the cellular synthetic machinery and causing the synthesis of specialized elements that can transfer the viral genome to other cells" (Vardiman, 2009). Results of previous works demonstrated the relevance of retroviruses in leukemia. The first human retrovirus identified was human T-lymphotropic virus or HTILV-1 which is known to cause adult T-cell leukemia (Luria *et al*., 1978).Viral associations in lymphoma and leukemia patients are becoming increasingly more common but no direct evidence of a viral role in ALL causation is present but an indirect route of viral involvement has been suggested (Hoelzer and Gökbuget, 2005). Samples from acute lymphoblastic leukemia (ALL), chronic

Table 1. Primers and their sequences

No.	Primer name	Type	Primer sequence	bp
1	EBV-BNLF1	Forward	5- CTA GCG ACT CTG CTG GAA AT- 3	380
2		Reverse	5- GAG TGT GTG CCA GTT AAG GT- 3	
3	EBV-EBER2	Forward	5- CCC TAG TGG TTT CGG ACA CA- 3	108
4		Reverse	5- ACT TGC AAA TGC TCT AGG CG- 3	
5	HHV6	Forward	5′ GAT CCG ACG CCT ACA AAC AC 3′	249
6		Reverse	5′ TAC CGA CAT CCT TGA CAT ATT AC 3′	
7	HHV8- 1	Forward	5′-CTA TCC AAG TGC ACA CTC GCT GTC C-3′	
8		Reverse	5′-GGA ACC AAG GCT GAT AGG ATA CAA AGG-3′	461
9	HHV8-2	Forward	5′-TTT GAT GGC GTC GGT CTC TAC C-3′	
10		Reverse	5′-CGC ACA TAT CGT CTG GAC GTA G-3′	524
11	HHV8-3	Forward	5′-GTG ATG TCA TCT GGG ACG CTC AAC-3′	
12		Reverse	5′-GTG AGA CAT CTC CGG ACT ACA TCC-3′	634

lymphoblastic leukemia (CLL) and Hodgkins lymphoma were found to be positive for Epstein-Barr virus (EBV) by polymerase chain reaction (Matsuoka and Jeang, 2007; Montserrat, 2005; Tsimberidou *et al.,* 2007). These results are suggestive that EBV, a B-lymphotropic human herpes virus and may be other viruses may play a role in the progression of some types of leukemia (Rolston and Bodey, 2010; Hasserjian, 2011). The current work was planned to detect the role of some herpes viruses in leukemia patients using serological and molecular methods.

MATERIALS AND METHODS

Subjects

The study was carried out from August 2012 till May 2013 in the laboratories of Genetic Engineering and Biotechnology Institute for Postgraduate Studies, University of Baghdad and Baghdad Teaching Hospital. Two enrolled groups, seventy five samples were taken from Iraqi patients newly diagnosed with leukemia aged between (2-74) years from Baghdad Teaching Hospital, The Protection of Children Hospital, AlKademeya Teaching Hospital and Central Children Hospital –Baghdad. The disease was clinically diagnosed by a consultant medical staff. Questionnaire form has been filled for each patient. Twenty five samples were taken from healthy people- as control- aged between (8-62) years and they had no history or signs of leukemia.

Blood sample collection

Peripheral blood (5ml) was obtained under aseptic conditions from each subject by a vein puncture using a disposable syringe. Blood samples were divided into two into two tubes, first tube: EDTA tubes for DNA isolation and second tube for serum samples. The serum obtained by putting the blood samples in a clean dry plain plastic tube and allowed to clot at 37 ºC for 30 minutes before centrifugation. The blood sample and serum were placed in a cool box and transferred to the laboratory, where it was kept at -20˚C and processed within 24h.

Serology Test for EBV

The MONO mononucleosis rapid test is a simple test that utilizes an extract of bovine erythrocytes to qualitatively and selectively detect infectious mononucleosis heterophile antibodies that caused by Epstein-Barr virus in serum in minutes. 25 µl of serum and 55 µl of buffer were transferred to specimen well of the device and set timer for 10 minutes and read the results.

Genomic DNA Isolation

Two methods were used for isolation of genomic DNA from leukemia patients and control. The first one by isolating the genomic DNA from human whole blood by Genomic DNA Mini Kit Geneaid/Korea, the other one was by isolating the viral nucleic acid from serum by QI Aamp Ultra Sens Virus Kit Qiageen/ Germany. DNA concentration was measured by nanodrop system (Bioneer/Korea).

Polymerase chain reaction (PCR)
Primers

Primers were provided by Alfa DNA Company / Canada in a lyophilized form which was diluted with sterile distilled water to a final concentration of 10pmol/ml. The primers and their sequences are listed in Table 1.

PCR Programs

DNA samples were subjected to PCR using master mix (Promega Corp., Madison, WI), primers and a thermal cycle (Applied Biosystem-USA). The standard cycle procedure as follow For EBV (BNLF1) was a 5-minute denaturation at 95 °C for one cycle, then 35 cycles of 30 seconds of denaturation at 95 °C, I minute of annealing 55°C, 2 minutes extension at 72 °C and 7 mins for final extension at 72 °C(Bonnet *et al*., 1999). For EBV (EBER2) was a 5-minute denaturation at 95 °C for one cycle, then 35 cycles of 30 seconds of denaturation at 95 °C, I minute of annealing 60°C, 2 minutes extension at 72 °C and 2 mins for final extension at 72 °C (Bonnet *et al*., 1999). For HHV6 was a 2.5-minute denaturation at 95 °C for one cycle, then 35 cycles of 30 seconds of denaturation at 95 °C, 30 seconds of annealing 62°C, 50 seconds extension at 72 °C and 5 mins for final extension at 72 °C (Collandre *et al.*, 1991). For HHV8 was a 2 minutes denaturation at 95 °C for one cycle, then 35 cycles of 1 min of denaturation at 95 °C, 1 min of annealing between 58°C, 45 seconds extension at 72 °C and 1 min for final extension at 72 °C (Foreman *et al*., 1998). The PCR products and the ladder marker were resolved by 2% agarose electrophoresis. The bands were visualized on UV transiluminator and then photographed by using photo documentation system.

Ethical use of data

Informed consent was obtained from all the study participants, and the guidelines set by the ethics committee of our institute and hospitals were applied.

RESULTS AND DISCUSSION

Subjects data

A total of 100 blood samples were collected from two Iraqi groups include leukemia patients and control. The total number of patients group was 75 while it was 25 for control group.

Age, gender, family history and geographical distribution of the samples

In this study 75 leukemia patients and 25 as control were subjected to serological and genomic analysis by PCR. Patients were distributed into four groups, acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphoid leukemia (CLL). The age of all leukemia patients was ranged less than 3 to more than 50years. Figure 1 revealed that 40% of the study leukemia patients were between (16 -30) years, followed by 24% of patients whose age ranged between (31-50) years, 18.7% of patients whose age (2-15) years and ends with 17.3% of patients whose age (50>) years. The male were 49.33% while females were 50.66%, as shown in Figure 2. Concerning patients group and control, both showed that negative family history recorded was higher than positive family history Figure 3. The most cases of leukemia patients were residents in

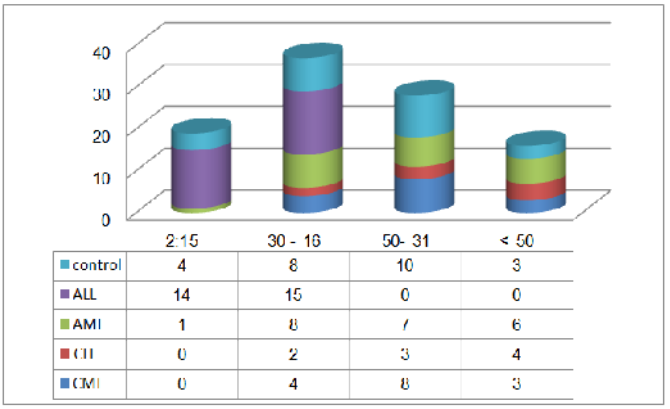

Figure 1. Distribution of leukemia patients and control group mean according to their ages

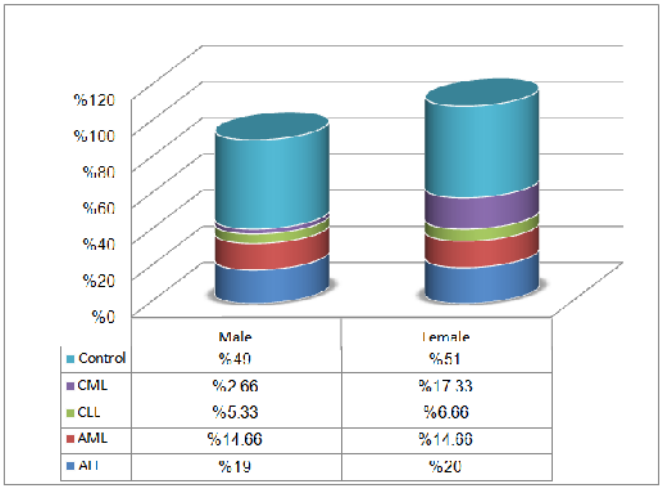

Figure 2. Distribution of leukemia patients and control according to gender

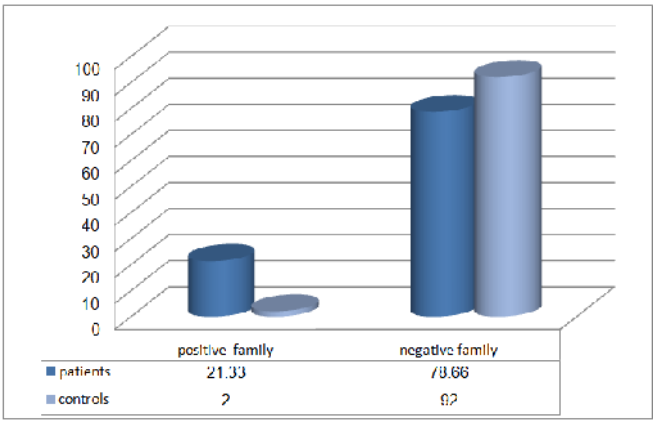

Figure 3. Distribution of leukemia patients and control groups according to family history

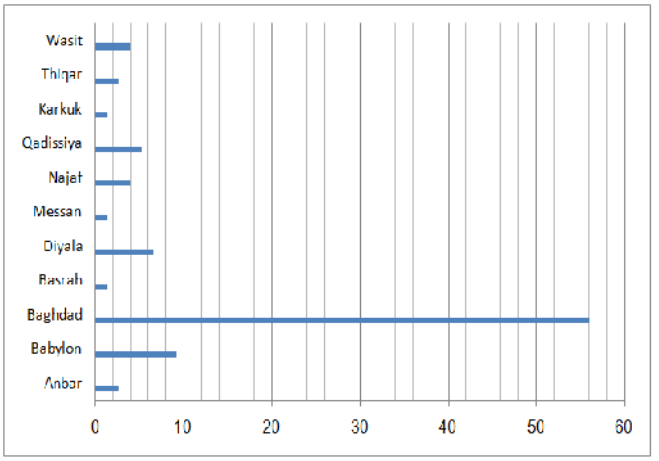

Figure 4. The Geographical Distribution of the Leukemia Patients Samples in most Iraqi Governorates

Baghdad 42 (56%) followed by Babylon 7 (9.3%), Diyala 5 (6.6%) and Qadissiya 4 (5.3%), Figure 4. In general, the reported results of this study showed that acute leukemia occurred at any age mainly in early childhood and a progressive rise in incidence was observed with age. Over all incidences, AML occurs in all age groups, while ALL is the most common leukemia of childhood. CML and CLL are rarely found in childhood, but occurred in adolescents and adults. In this study we had shown that the occurrence in leukemia patients was in the following order: ALL, AML, CML and CLL. The small difference between the ratios of male and female may be due to the small number of cases involved in this study or the difficulties in attending Baghdad specialized hospitals from different governorates, specially, for female due to the present improper security situation. The pre-rate in Baghdad showed a higher number related to the large population in this town besides the present unstable security situation which greatly affects traveling of patients from remote area. The geographical distribution of the patients groups reflects that Leukemia could affect different Iraqi regions a fact that might indicate that the disease could occur in all regions, races and ethnic groups.

Serological Examination

Among 100 serum samples (75 patients+25 control) two patients samples were found to have a positive results for EBV (2%) belong to ALL and AML patients (one patient each).

PCR analyses

118 DNA samples (75 from blood and 43 from serum) beside 29 samples from controls (25 from blood and 4 from serum) were analyzed by PCR. In the first category, no amplified products were detected for BNLF1, HHV6, HHV 8-1, HHV8-2and HHV8-3 primers. The failure of these primers to amplify the genomic DNA of leukemia patients and healthy may be attributed to the absence of suitable priming sites for these primers in the genomes of tested leukemia patients and healthy individuals. In other words, there are no complementary sequences for these primers in leukemia patients and normal individual's genomes but that not mean that negative PCR products mean absent of viruses. Nefzi, 2010referred that confirms the absence of role of HHV-6 in the genesis of acute leukemia but the virus was reactivated after chemotherapy treatment. HHV-6 was more prevalent in the blood samples from children than from adults (25% and 9%, respectively). A case of HHV-6 chromosomal integration was detected in one patient with AML. On the other hand, different rates of HHV-8 infection have been reported in various populations in the world .The prevalence of HHV8 in healthy individuals was found to be 1.3%-4.4% in Southeast Asia and the Caribbean regions and > 40% in Africa (Nefzi *et al.*, 2012; Ablashi *et al.*, 1999**)**.

In India a prevalence of 3.7% and 2.3% has been reported in healthy individuals and HIV positive patients, respectively **(**Sitas *et al*., 1999**)**. In one study from Saudi Arabia the seroprevalence of HHV-8 in healthy Saudi national's people was reported to be 1.7% **(**Alzahrani *et al*., 2005**)**. In Europe, the prevalence of HHV-8 was found to be lowest in Spain or Greece (6%-8%) and highest in Italy (20.4%) **(**De Sanjose *et al.*, 2002; Zavitsanou *et al*., 2007**)**. Approximately 50% of the adult population of Brazilian Amerindians was reported to have antibodies to HHV-8 **(**Biggar *et al.*, 2000**)**, compared with only 11% of HIV negative injection drug users in Argentina **(**Sosa *et al.*, 2001**)**. Whereas primers in the second category were found to produce amplified products, this category included one primer, namely EBER2**.** When using this primer, a reasonable degree of DNA polymorphism was detected only in leukemia patients.

Primer EBER2

The presence of Epstein–Barr virus was detected in the fourteen samples by checked both serum and blood for the same sample. Ten samples appeared positive with serum and two showed positive results in blood only and only two sample showed positive results in serum and blood (these samples were showed positive result with the serology test too) (Table 2).

Table 2. EBV EBER2 positive PCR production blood and serum samples.

Samples	PCR + ve to EBV	Type of sample		
		Blood only	Serum only	Both serum and blood
ALL	9	2	6	1
AML	2	-	1	1
CLL	3	-	3	-
CML	-	-	-	-
Control	-	-	-	-
Total	14	2	10	2

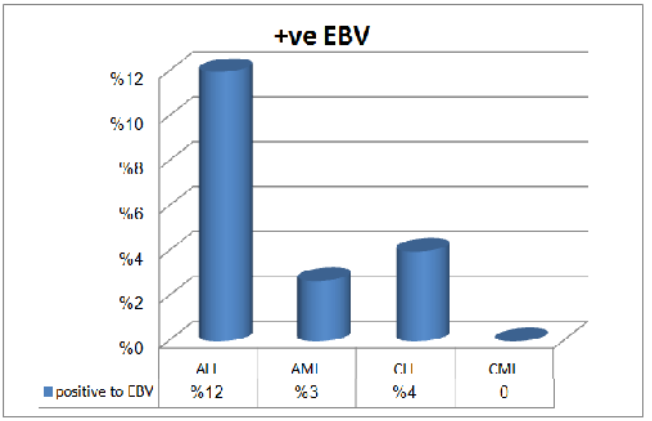

Figure 5. The number of the positive samples to EBV in Leukemia samples and control

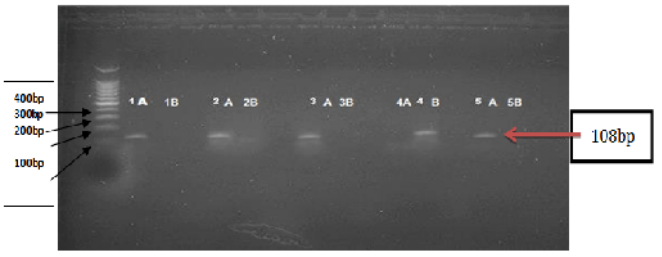

Figure 6. PCR products of EBV EBER-2 on 2% agarosegel at50 voltages for (60) min.A = serum sample, B = blood samples

The fourteen positive samples were divided as: nine samples of ALL, two sample of AML and three samples of CLL, while no PCR product was detected in CML samples (Figures 5 and 6). Detection of EB virus in leukemia patients was also detected by Omer *et al*., (2011) who reported that EBV infections are more prevalent among leukemic patients. There was an increase in the seropositivity rates of EBV infections with increasing ages of leukemia patients. The male leukemic patients were more exposed to EBV infections than females. The presence of Epstein–Barr virus were detected in the fourteen samples by checked both serum and blood for the same sample, ten samples appeared positive with serum and two showed positive results in blood only, and only two sample showed positive results in serum and blood and these samples were showed positive result with the serology test too Table 2.

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