Detection of Epstein–Barr virus and Cytomegalovirus in Iraqi Acute Lymphoblastic Leukemia patients

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الخلاصة
شملت هذه الدراسة 50 مريضا تم تشخيص إصابتهم بسرطان الدم الليمفاوي الحاد تراوحت أعمارهم من 5 إلى 45 سنة بالإضافة إلى 50 فرد من الأشخاص الأصحاء من أعمار متناظرة اعتبرت مقياسا للسيطرة
تم تحديد نسبة حدوث الإصابة بفيروس ابشتاين بار EBV و الفيروس المضخم للخلايا CMV لدى المرضى المصابين بسرطان الدم الليمفاوي الحاد عن طريق الكشف عن المستويات المصلية للغلوبيولينات المناعية (غلوبيولين المناعي G ، غلوبيولين المناعي M) لكليهما بطريقة فحص مقايسة ألماز المناعي للأنظيم المرتبط ELISA وطريقة الأضداد المتألقة بالإضافة إلى الكشف عن الاحماض النووية الفيروسية لكليهما بطريقة المقايسة المعتمدة على (CISH) وأشارت النتائج ان 24٪ من مرضى سرطان الدم الليمفاوي الحاد كانوا مصابين بفيروس ابشتاين بار EBV و 28٪ منهم أيضا كانوا مصابين بفيروس مضخم الخلايا CMV.

Abstract
Objective : determination the percent of infection of EBV and CMV infections in Iraqi patient infected with ALL .Methods : the study have concentrated to determine the rate of EBV and CMV infections by detecting EBV and CMV antibodies in both the IgM and IgG classes of immunoglobulins by ELISA method and Indirect Immunofluorescence Test (IIFT) as well as chromogenic in situ hybridization (CISH) for detecting Epstein-Barr virus (EBV) EBER RNA and CMV DNA . Results : from total 50 ALL patients were involved in this study , 50% were having viral infection (22% patients infected with EBV and 28% infected with CMV) this may be referred that in present study the viral causes may be equal to other unknown etiological factors of ALL collectively. anti-EBV Ig G and EBER RNA were detected in(24%,12) of all ALL patients and anti EBV Ig M was found only in (4%,2) of ALL patients and patients with positive EBV Ig M also were positive for anti EBV Ig G and EBER RNA while anti-CMV IgG and CMV DNA were detected in (28%,14) of all ALL patients involved in the study and only (8%,4) of ALL patients showed positive anti CMV Ig M and patients with positive anti CMV Ig M also were positive for anti CMV Ig G and CMV DNA. Conclusion : The presence of elevated levels of EBV-infected cells or CMV-infected cells within the peripheral blood may be risk factors for developing ALL and the incidence of CMV infection (28%) was slightly greater than the rate of EBV infection (24%) in ALL patients and this may be referred that in present study the viral causes may be equal to other unknown etiological factors of ALL collectively.

Introduction
Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer. It is a type of cancer that starts from white blood cells in the bone marrow called lymphocytes (1) . where it accounts for about 75% of childhood leukemia and 25% of all pediatric cancer (2) it is slightly more common among white children and boys through 14 years of age (3**)** but also seen in

Viruses can cause benign or malignant tumors in many species of animal and human viral infection by at least one known virus human T-cell

leukemia/lymphotropic virus type I (HTLV-1) is a well-understood cause of adult T-cell leukemia. (4 , 5)

Epstein –Barr Virus (EBV) and Cytomegalovirus (CMV) establishe a life long latency that is clinically asymptomatic the clinical and biological evidence indicates that they may disrupt this latency and become causally linked to several tumors (6 , 7.

Also apoptosis is an antiviral defense mechanism by which the host can eliminate infected cells and restrict viral propagation. To overcome this response, EBV and CMV encode proteins that prevent or attenuate apoptosis in infected cells. (8)

Evidences that EBV and CMV are truly the causative agents exist in many previous studies, EBV is the cause of several tumors especially those of lymphoid and epithelial origin (9 , 10)

Patients and Methods

This study was achieved on 50 patients were diagnosed with acute lymphoblastic leukemia , their age ranged from 5 to 45 years and 20 healthy individual with matched age as control group attending to Baghdad teaching hospital, teaching laboratories and Pediatric Oncology Unit at the Al-Mansour teaching hospital in Baghdad medicine city and center of blood diseases in Baghdad from different provinces of Iraq . study was conducted in the period between beginning of February / 2011 to the end of April / 2012.. All patients were not under medication and without family history .

Methods : EBV and CMV infections in ALL patients have depended upon detecting EBV and CMV antibodies in both the IgM and IgG classes of immunoglobulins by ELISA method and Indirect Immunofluorescence Test (IIFT) as well as chromogenic *in situ hybridization*(CISH) for detecting EBER RNA and CMV DNA (according to company instruction) .

EBV IgG and IgM is an ELISA kite for quantitative determination of IgG and IgM against capsid antigen to Epstein Barr Virus in human serum (according to Human company instruction).

CMV IgG and IgM is an ELISA kite for quantitative determination of IgG and IgM against CMV antigen in human serum (according to Biocheck company instruction).

IIFT : is an in vitro assay for the determination of specific Ig M or Ig G antibody against CMV antigen or EB-VCA , Biochips coated with CMV-infected cells are fixed onto the reaction field of a microscope slide with Euroimmun Biochips technology. In the case of positive reactions specific antibody of class Ig G and Ig M will bind to the viral antigens. In a second step the attached antibody are stained with fluorescein-labelled anti human antibodies and made visible with the fluorescence microscope.

CISH : the presence of certain nucleic acid sequences in Smear of lymphocytes isolated by lymphprep was done for each ALL patient and the smears were fixed in absolute methanol (modified step in this study) can be detected by in situ hybridization using labeled DNA probes .The hybridization results in duplex formation of sequences present in the test object with the labeled DNA probe Duplex formation of the digoxigenin-labeled probe with Epstein-Barr-Virus (EBV) EBER RNA in the test material is indirectly detected by using enzyme conjugated antibodies directed against digoxigenin detected by a secondary polymerized enzyme-conjugated antibody. The enzymatic reaction of a chromogenic substrate leads to the formation of a color precipitate that is visualized by light microscopy (

according to Bremerhaven company instruction, Germany)

Results

Results were represented in figure -1 showed that from total 50 ALL patients 50% were having viral infection (22% patients infected with EBV and 28% infected with CMV) this may be referred that in present study the viral causes may be equal to other unknown etiological factors of ALL collectively.

The results of immunologic screening and virologic nucleic acid data for detection of EBV and CMV in 50 ALL patients are summarized in tables -1 and -2 respectively. Anti-EBV Ig G and EBER RNA were detected in (24%,12) of all ALL patients involved in this study and anti EBV Ig M was found only in (4%,2) of ALL patients and patients with positive EBV Ig M also were positive for anti EBV Ig G and EBER RNA as were explained in table -1 while anti-CMV IgG and CMV DNA were detected in (28%,14) of all ALL patients involved in the study and only (8%,4) of ALL patients showed positive anti CMV Ig M and patients with positive anti CMV Ig M also were positive for anti CMV Ig G and CMV DNA as were shown in table -2. Positive results for detection Ig M against EB-VCA by IIFT method were illustrated in figure -2 in addition positive reactivity for (EBV) EBER RNA in the lymphocytes by CISH method was explained in figure -3 In present study , the incidence of CMV infection 14(28%) was slightly greater than the incidence of EBV infection 12(24%).

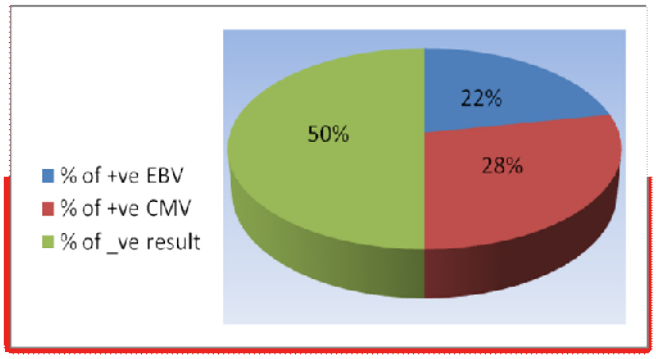

Figure (1) : % of EBV and CMV infection in 50 ALL patients

Table -1: Results of detection serum EBV IgM , EBV IgG antibody in ELISA and IIFT as well as EBER RNA in CISH of 50 ALL patients

ELISA EBV Ig G	ELISA EBV Ig M	IIFT EBV Ig G	IIFT EBV Ig M	CISH EBER RNA	Number of patients	% of patients
+	+	+	+	+	2	4%
+	_	+	_	+	10	20%
_	_			_	38	76%
Total					50	100%

Table -2 : Results of detection serum CMV IgM , CMV IgG antibody in ELISA and IIFT as well as CMV DNA in CISH of 50 ALL patients

ELISA CMV Ig G	ELISA CMV Ig M	IIFT CMV Ig G	IIFT CMV Ig M	CISH CMV DNA	Number of patients	% of patients
+	+	+	+	+	4	8%
+	_	+	_	+	10	20%
_	_	_	_	_	36	72%
Total					50	100%

CISH (chromogenic in situ hybridization)
ELISA(Enzyme Linked Immunosorbent Assay)
IIFT (Indirect Immunofluorescence Test)

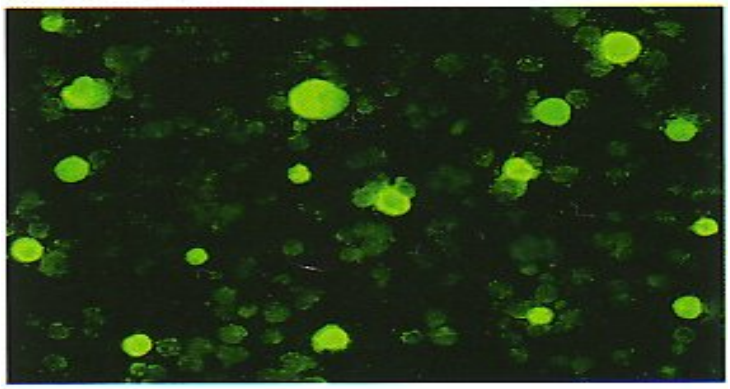

Figure -2 : IIFT slide of positive Ig M antibody against EB-VCA (magnification x1000)

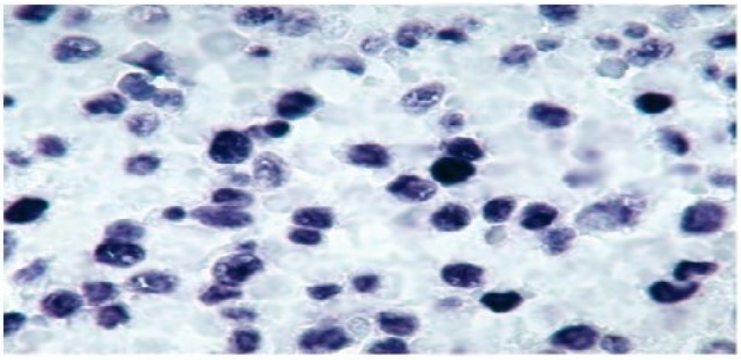

Figure -3 : CISH analysis of a positive reactivity for (EBV) EBER RNA in the lymphocytes is indicated by a distinctly stained dark violet-blue nucleus which clearly distinguished from the background when using NBT/ BCIP as substrate (magnification X1000

Discussion

The study have attempted to determine the incidence of EBV and CMV infections in ALL patients to relate infections to some of the clinical complication seen in patients with this infections and to evaluate laboratory methods for determining primary and secondary EBV and CMV infections in ALL patients.

Results represented in this study showed that from total 50 ALL patients 50% were having viral infection (22% patients infected with EBV and 28% infected with CMV) this may be referred that viral causes may be equal to other unknown etiological factors of ALL collectively. So, we here in briefly discus the suggesting a possible role of EBV and CMV as a direct or indirect

microenvironmental progression factor in ALL.

The reported incidence of EBV and CMV infections in ALL patients have depended upon detecting EBV and CMV antibodies in both the IgM and IgG classes of immunoglobulins by ELISA method and IIFT as well as chromogenic in situ hybridization(CISH) for detecting EBER RNA and CMV DNA tests have yield no false positive results or non specific cross reactions and was more readily performed furthermore . The study believe that they were preferable and more useful in diagnosis of primary EBV or CMV infection. Since IgM be highly elevated in comparison with IgG which is either absent or in the begging in primary immune response, it could be possible to determine a primary infection by noting the rising of IgM that lead to differentiate from the rising of IgG in past duration or chronic infection (11)

The presence of anti CMV IgM antibody in 8% ALL patients infected with CMV may be related to that CMV is important latent infection and the role of chemotherapy in these ALL patients could not be ignored , also CMV has a specific mechanism of immune evasion that allows latent state in leukocytes for long periods and can be reactivated when cell-mediated immunity is decreased .(12) CMV being latent and opportunistic infection due to therapy or immunosuppression therefore , it is quite possible that CMV by itself may not cause ALL and may be an important cofactor at least in some patients (13)

In present study , the incidence of CMV infection 14(28%) was slightly greater than the incidence of EBV infection 12(24%) in ALL patients, the reason for this higher incidence it may be secondary to the immunosuppression therapy that precedes the infection with CMV during the course of ALL

Recently, malignancies linked to oncogenic viruses EBV and CMV often demonstrate resistance to apoptosis, although the specific mechanisms through which viruses directly or indirectly prevent cell death programs within tumor cells remain elusive. (14) .

EBV expresses a set of latent proteins among which is latent membrane protein 1 (LMP1) is able to transform numerous cell types and is considered the main oncogenic protein of EBV. The mechanism of action is itself has been implicated in host cell resistance to apoptosis through NF-B-mediated up-regulation and act as anti-apoptotic molecules (15). Also EBV latent membrane protein 1 (LMP-1) protects infected B cells from apoptosis by up regulation of the bcl-2. EBNA-2, another EBV latent protein, can increase the effect of LMP-1 on B cell lymphomas (bcl-2) expression. Bcl-2 is anti-apoptotic molecule first discovered in follicular B cell lymphomas.(16)

Louise et al., 2009 (17) identified a human cytomegalovirus cell-death suppressor denoted vICA encoded by the viral UL36 gene that encodes the viral inhibitor of caspase-8 activation (vICA) . vICA inhibits Fas-mediated apoptosis by binding to the pro-domain of caspase-8 and preventing its activation, caspase-8 activates extrinsic apoptosis and also functions to promote monocyteto-macrophage differentiation.

EBV transforms B lymphocytes in culture and causes lymphomas in marmoset monkey it is also associated with nasopharyngeal carcinoma, tumor that occurs primarily in China and with thymic carcinoma and B-cell lymphoma in the United States. Also cells isolated from East African individuals with Burkitt,s lymphoma contain EBV DNA and EBV nuclear antigen(18) only a small fraction of many copies of EBV DNA is integrated most viral DNA is in the form of closed circles in the cytoplasm ,the difficulty in proving that EBV is a human tumor virus is that infection by the virus is rare and the current hypothesis is that EBV infection induces B cells to proliferate, thus

increasing the likelihood that a second event such as activation of a cellular oncogene c-myc which is normally located on chromosome 8, is translocated to chromosome 14 at the site of immunoglobulin heavy chain genes. This translocation brings the c-myc gene in juxtaposition to an active promoter ,and large amounts of c-myc RNA are synthesized .it is known that the c-myc ooncogene encodes a transcription factor but the role of this factor in oncogenesis is uncertain . (19).

The final outcome of the interaction between EBV and the infected host is the establishment of a nonpathogenic latent infection of memory B lymphocytes that allows the virus to persist for the lifetime.

EBV can be considered as the prototype of oncogenic viruses (20) that behave as direct transforming agents. In fact, in classical EBV-associated tumors, the virus genome is present in virtually all neoplastic cells, which show the expression of viral RNAs and proteins that variously contribute to the induction of the transform phenotype. (21). According to these features and of the strict association with distinct tumor types, EBV has been classified as a group I carcinogen. An additional compelling factor is the presence of homogeneous (clonal) EBV episomes detected with the use of the virus termini assay in several EBV-related tumors (HL, NPC, BL) as well as in some pre-neoplastic lesions (22 , 23). These findings suggest that these tumors develop from a single cell that was infected by EBV before the outgrowth and are consistent with a role for EBV in the early phases of tumor development besides the well defined group of tumors pathogenically. associated with EBV according to the criteria mentioned above. The presence of this herpesvirus has been variably detected in a broad spectrum of other tumors for which a causal role of EBV seems unlikely. These tumors include also chronic lymphocytic leukemia. (24)

Besides behaving as a direct drive of neoplastic progression in EBV-infected ALL cells, the presence of EBV within tumor microenvironment could also indirectly contribute to the malignant evolution of the disease. Indeed, EBV may infect a subpopulation of ALL cells and/or may be carried by stander normal B lymphocytes EBV infection

Regarding to CMV, in previous study human CMV can be disrupted HLA class 1 expression by preventing maturation assembly and migration of the trimolecular MHC complex(25 , 26) similar mechanisms apply for MHC class 11 molecules (27 ,28) and the primary goal of established HCMV stealth features is the quantitative downregulation of the bulk of MHC class I molecules from the cell surface (29 ,30) because antigen presentation to CD8+ T cells is a major defense mechanism against virally infected cells. The major histocompatibility complex class I (MHC I) antigen presentation pathway exposes peptide antigens on the cell surface to surveilling CD8+ T cells. Upon a fitting contact between the T cell receptor and an MHC I-peptide complex, the CD8+ T cell becomes activated to induce lytic destruction of the recognized target cell (31) therefore, the down regulation of HLA class 1may disrupt CD8+ T cell recognition.

Recommendations

1- We recommend to depend CISH method for detection EBV and CMV because it gather both molecular and immunological detection for them with high specificity and sensitivity.

2- Further work is needed to clarify the specific role of some immunological parameter such as cytokines like IL-1, IL-2, IFN-γ and IL-12 in the persistency of the EBV and CMV viruses.

3- Further studies are required to detection other possible etiological viruses such as Hepatitis B virus(HBV), Hepatitis C virus (HCV) and Human papillomavirus (HPV)

4- Further studies are required to prepare vaccines against EBV and CMV.

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