Original article

Association of human herpesvirus 6 with lymphoid malignancies in Iraqi patients

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ABSTRACT:

Background: Human herpesvirus type 6 (HHV-6) is associated with roseola infantum during childhood followed by life-long latency that periodically reactivated in immunocompromised individuals**.** In spite of several studies to establish the pathogenic role of HHV-6 in lymphoid malignancies, the issue is still controversial.

Objectives: This study was arranged to explore the association of HHV-6 infection in lymphoid malignancies using different serological and molecular techniques and to quantify the plasma viral load.

Patients and methods: This cross-sectional case control study was conducted in National Center for Hematological Diseases (NCHD) at Al-Mustansiriyah University and Baghdad Teaching Hospital (BTH) in Baghdad-Iraq from September 2013 till April 2015. The patient group consists of 11 patients with Hodgkin lymphoma and 39 Non-Hodgkin's lymphoma of both sexes. The age range was between 15-80 years. The diagnosis of lymphomas was based on hematological and histopathological criteria. 59 apparently healthy individuals were enrolled as control group. They were chosen from unpaid blood donors. The age range was between 18-59 years. Human privacy was respected by taken participant's oral consensus. The seropositivity rate of anti-HHV-6 IgG and IgM antibodies were detected by enzyme linked immunosorbent assay (ELISA) and indirect immunofluorescent test (IFAT). The molecular detection and determination of plasma viral DNA load was achieved by quantitative polymerase chain reaction (qPCR). All data were statistically analyzed, and P values < 0.05 were considered significant.

Results: The anti-HHV-6 IgG positivity rate by IFAT was insignificantly higher in HL (81.8% *vs* 61.0% p=0.186) and NHL (64.1% *vs* 61.0%, p =0.758) compared to control group. The anti-HHV-6 IgG positivity rate by ELISA was 81.8% in HL, 84.6% in NHL versus 72.9 % in controls which were insignificant in both groups (p=0.534 and p=0.173) respectively. The anti-HHV-6 IgM positivity rate by ELISA technique among patients with HL was significantly higher compared to controls (27.2% *vs* 6.8%, p= 0.038), but not significant in NHL (17.9% *vs* 6.8%, p= 0.086). HHV-6 DNA was detected in (27.3%) patients with HL by PCR technique, but none of the controls or NHL patients was positive. The plasma viral DNA load of the patient with HL was $1.4\pm 0.3 \times 10^5$ copies/milliliter.

Conclusion: Although a higher anti-HHV-6 antibodies positivity rate among patients with HL and NHL, the pathogenic role of the virus in the development of these malignancies was difficult to be ascertain.

Keywords: Human herpesvirus-6, lymphoid malignancies, Hodgkin's lymphoma

Introduction:

The subfamily *Beta herpesvirinae* contain lymphotropic viruses that have a lesser confined cell tropism including HHV-6, which belong to the Roseolovirus genus [1,2]. The HHV-6 was wide prevalent virus as the transmission occurs easily via saliva and air droplets. The primary infection of HHV-6 established latency, most expected in macrophages and/or monocytes [3,4]. Viral reactivation may lead to severe secondary complications particularly in immunocompromised patients as those of bone marrow transplantation [5,6].

HHV- 6 was divided into 2 subtypes; HHV-6A and HHV-6B [7]. HHV-6A involves many strains derived from adult and many researchers thought that the virus more *neuroinvasive* [8,9]. HHV-6B is etiologic agent of roseola infantum, in children; despite the fact that the two viruses have 95% homology in their sequence [10].

Reactivation initiate periodically in immunocompetent carrying the virus in latent stage and the reactivation was asymptomatic, but serious complication can be occurred in immuneocompromised individuals [11]. The HHV-6 may have a role in malignancies directly by the ability of HHV-6 to infect CD_4^+ T-cells and induce apoptosis and indirectly may contribute to cancer by immune suppression [12]. Currently it became known that HHV-6 can also infect hematopoietic stem cells (HSCs), epithelial cells of the thymus and natural killer cells (NK) and the later have great magnitude for immune maturation as well as protection against cancer and viral infections. Therefore, the active infection of HHV-6 can promote pathologic property of other viral infections [13,14,15].

Previous reports concerning HHV-6 positivity of HL have resulted in conflicting findings. However, an association between the virus and nodular sclerosis subtype of Hodgkin's lymphoma (NSHL) has been documented by many investigators by different laboratory techniques [16]. It was reported that a higher rate of HHV-6 DNA in series of angioimmunoblastic T cell lymphoma (AITL), which is a subtype of T-cell non-Hodgkin's lymphoma was well characterized, in contrasted with other subtypes of lymphoma and controls [17]. Furthermore, a clear association between histological progression of AITL and the detectable copy number of both EBV and HHV-6 B in the AITL tissue specimens was confirmed [18].

Patients and methods:

This prospective cross-sectional study was carried out at the NCH at Al-Mustansyria University and BTH in Baghdad-Iraq from September 2013 till April 2015. The patients group consists of 11 patients (3 male and 8 female) with HL and 39 NHL (21 male and 18 female) and the control group include 59 apparently healthy individuals, randomly selected from unpaid blood donors attending the NBB in Baghdad. The age range of patients with HL was 17-70 years, 20-80 years in NHL and 18-59 in healthy controls. Diagnosis of these malignancies was based on hematological, bone marrow and histopathological criteria. Ten milliliter of venous blood samples were withdrawn aseptically from patients and controls and the blood sample was divided in two parts first with EDTA tube for plasma separation and the second in plane tube for serum separation.

Both serum and plasma sample were stored in aliquots at -80 ^{0}C. Detection of anti-HHV-

6 IgG antibodies was done by ELISA (Abnova company - Taiwan) and by IFAT (VIDIA company,Czech Republic) while the anti-HHV-6 IgM was detected by ELISA (Abnova company - Taiwan) . The HHV-6 A-B genome was quantified using the real -time PCR for the (Genetic PCR Solutions TM, Spain). Another PCR kit was used for titration HHV-6 dtec-qPCR DIA.PRO (Diagnostic Bioprobes, Italy). Human privacy was respected by taken participant's oral consensus. Data were analysed using the SPSS-22 (Statistical Packages for Social Sciences- version 22).

Results:

The anti-HHV-6 IgG positivity rate by IFAT among HL patients and controls. 9 (81.8%) patients and 36 (61.0) controls. The difference between the two groups was statistically insignificant (p=0.186), Table (1).

On the other hand, the anti-HHV-6 IgG positivity rate among HL patients using ELISA was 81.8%, while that of the controls was 72.9 %, the difference between the two groups was statistically insignificant (p=0.534), table (2).

The anti-HHV-6 IgM positivity rate as detected by ELISA technique among patients with HL was significantly higher compared to controls (27.2% versus 6.8%), (p= 0.038), Table (3).

Table (1): Number and percentage of anti-HHV-6 IgG in HL patients compared to control group by IFAT test.

IFAT	HL		Control	
	No.	%	No.	%
Positive	9	81.8	36	61.0
Negative	2	18.2	23	39.0
Total	11	100	59	100
P=0.186 (No significant difference between proportions using Pearson Chi-square test at 0.05 level)				

Table (2): Number and percentage of anti-HHV-6 IgG in HL patients compared to control group by ELISA.

ELISA	HL		Control	
	No.	Percent %	No.	Percent %
Positive	9	81.8	43	72.9
Negative	2	18.2	16	27.1
Total	11	100	59	100
P=0.534 (No significant difference between proportions using Pearson Chi-square test at 0.05 level.				

Table (3): Number and percentage of anti-HHV-6 IgM in HL patients compared to control group by ELISA.

ELISA-IgM	HL		Control	
	No.	%	No.	%
Positive	3	27.2	4	6.8
Negative	8	72.7	55	93.2
Total	11	100	59	100
P=0.038 (Significant difference between proportions using Pearson Chi-square at 0.05 levels.				

The HHV-6 DNA was detected in 3 (27.3%) patients with HL, while none of the controls showed positive result. The plasma viral DNA load of the patient with HL was 1.4± 0.3 x10^5 copies/milliliter.

Table (5) showed that the anti-HHV-6 IgG positivity rate among NHL patients using IFAT was 64.1%, and that of the controls was 61.0%. The difference between the two groups was statistically insignificant (p=0.758).

Using the ELISA, the anti-HHV-6 IgG positivity rate among NHL patients was 84.6% versus 72.9% in the controls, (Table 6). Again the difference between the two groups was statistically insignificant (p=0.173).

Table (7) revealed that the anti-HHV-6 IgM positivity rate as detected by ELISA technique was higher among patients with NHL (17.9%) compared to controls (6.8%). However, the difference was failed to reach the levels of statistical significance (p= 0.086).

Table (4): Number and percentage of HHV-6 DNA in HL patients compared to control group by PCR.

PCR	HL		Control	
	No.	%	No.	%
Positive	3	27.3	0	-
Negative	8	72.7	59	100
Total	11	100	59	100

Table (5): Number and percentage of anti-HHV-6 IgG in NHL patients compared to control group by IFAT test.

IFAT	NHL		Control	
	No.	%	No.	%
Positive	25	64.1	36	61.0
Negative	14	35.9	23	39.0
Total	39	100	59	100
P=0.758 (No significant difference between proportions using Pearson Chi-square test at 0.05 level.				

Table (6): Number and percentage of anti-HHV-6 IgG in NHL patients compared to control group by ELISA.

ELISA	NHL		Control	
	No.	%	No.	%
Positive	33	84.6	43	72.9
Negative	6	15.4	16	27.1
Total	39	100	59	100
P=0.173 (No significant difference between proportions using Pearson Chi-square test at 0.05 level)				

Table (7): Number and percentage of anti-HHV-6 IgM in NHL patients compared to control group by ELISA.

ELISA-IgM	NHL		Control	
	No.	%	No.	%
Positive	7	17.9	4	6.8
Negative	32	82.1	55	93.2
Total	39	100	59	100
P=0.086 (not Significant difference between proportions using Pearson Chi-square at 0.05 levels.				

Discussion:

In spite of higher positivity rate of HHV-6 IgG detected by IFAT in our HL patients versus healthy controls, there was insignificant difference between the two groups. Similar results were previously reported in HL patients using IFAT [19]. In another study, the HHV-6 IgG antibody titer was found to be elevated in relapsed HD patients post therapy in comparison with patients who did not [20]. Further analyses by [21] found that increased HHV-6 seropositivity is associated with ratio of geometric mean titer in HD young adults lacking social contact in the family group that may refer to late exposure to HHV-6 in those patients, suggesting that HHV-6 must be incorporated in additional explorations of the etiology of HD.

Using the ELISA technique, again our results showed higher but insignificant positivity rate of anti-HHV-6 IgG among HL patients versus healthy controls. These results are versus the results of previous study which found significant differences in HHV-6 seropositivity rate and titer of antibodies between patients with HD and low-grade NHL in compared to normal controls [21]. In the UK, a study investigated case clustering searching for EBV- Reed–Sternberg cell status by detection of EBV and HHV-6 serologic results, found that higher anti-HHV-6 antibody titers was primarily in patients with Reed–*Sternberg* cells negative for EBV, suggesting an etiologic exposure for HD independent from EBV [22]. The high prevalence of antiHHV-6 IgG in the general population clearly documents the wide circulation of this lymphotropic virus that may indirectly contribute to the pathogenesis of the lymphoproliferative disorder [23]

Our results show that there was significant increase in the levels of anti-HHV-6 IgM among patient with Hodgkin's

lymphoma compared to healthy subjects. In this context, our findings are in agreement with previous report in Belem, Brazil which examined a total of 323 patients with lymphadenopathy who were selected and screened for the presence of HHV-6 IgM by ELISA and the results found that 25% of lymphadenopathy cases were positive for HHV-6 IgM antibodies [24]. Although most viral lymphadenopathy is caused by EBV infection, CMV and HHV-6 are rare causes of mononucleosis in approximately 5% of cases [25,26]. Moreover, it has been reported that 3 patients with cervical lymphadenopathy were exhibited an IgM response or a high IgG titer to HHV-6 [27]. On another hand, two studies by [28] showed that 5% of normal adults may have detectable IgM.

The HHV-6 DNA detection rate was significantly higher among HL patients in our results. Similar results were reported by previous studies; in one of these the HHV-6 DNA was recorded in 13/45 (28. 8%) biopsies tissue samples from HD by nested PCR, even though no positive cases were discovered by blot. In another study, 12% of HD patients were positive for HHV-6 DNA by PCR [29,30] investigated both plasma samples and WBC's from patients with HL or NHL for detection of HHV-6 DNA and CMV DNA by PCR technique beside determination of the serum CMV antibody titer , 46% were positive for herpesvirus DNA (HHV6 or CMV) in WBC's or plasma which was significantly higher compared to pediatric control group, and of these 43% had active CMV infection, concluded that the presence of HHV-6 can be considered as a predicting indicator of cellular immunosuppression preceding the onset of CMV infection which may result in a severe outcome among pediatric lymphoma patients. Detection of HHV-6 DNA in lymphoid cells was another line of researches, and in this regard several other studies had yielded variable results;[31] used PCR for detection of HHV-6 DNA in lymph node specimens of 52 patients with HL, and found that 73 % were positive versus 68.4% positive in the control group. Related results were found that the HHV-6 DNA was integrated into host DNA of lymphoma cells [32]. In another study, the HHV-6 DNA was found in 16.6% in lymphocytes and histiocytes and occasionally in Hodgkin and *Reed-Sternberg* cells [33]. Additionally, using qPCR in lymph node specimens of 86 patient with HL found that 79.1% were positive for HHV- 6 genome, and the positive result was observed most often in the nodular sclerosis group (83.6%) of positive cases [34].

Keeping in the same line, [35] used PCR in lymph node specimens, and found that 13 % of patient with HL was positive for HHV6 DNA. While another study used quantitative PCR in lymph node biopsy found that 35.1% were positive for HHV-6 DNA, with all Hodgkin's lymphoma patients infected with HHV-6 presented with the nodular sclerosis subtype [36]. The later finding was supported by a study which found that 86% of nodular sclerosis HL (NSHL) had positive HHV-6 DNA [16]. Obviously, these studies and others offer arguments in favor of an implication of HHV6 in NSHL [37]. On the contrary, in minority of studies, HHV6 DNA was failed to be detected in HL patients [38].

Taken together, the common feature of these studies and ours is the high detection rate of HHV6 in HL patients; however, discrepancies in the detection rates may be attributed to many factors including

the type of specimens used, type of PCR employed and sample size. Of note, in our study, the real-time PCR was applied on plasma samples. Undoubtedly, beside the opportunity to measure the viral load, the recognition of HHV6 genome in plasma samples was considered as a right indicator of active viral infection and better correlates with clinical outcomes particularly when the plasma viral load was high [30,39].

Although the HHV-6 IgG positivity rate was elevated among NHL patients compared to healthy group; the difference between the two groups was statistically insignificant. These results are consistent with a previous results of a study documented that higher IgG positivity rate among NHL by IFAT [19]. Nevertheless, the current results are inconsistent with that reported a significantly higher HHV-6 IgG among lymphoma/myeloma versus healthy controls [23]. This discrepancy may be related to the fact that more than one type of blood malignancies was included in that study. Using the ELISA technique for detection of HHV-6 IgG, again our results revealed an insignificantly increase in positivity rate among NHL versus healthy control. These results are agree with prior studies documented a higher but insignificant increase of HHV-6 IgG in NHL compared to normal subjects [19,20].

The positivity rate of HHV-6 IgM in our results as detected by ELISA technique was found to be non-significantly higher in NHL patients versus healthy controls. Unfortunately, studies concerning the HHV-6 IgM in NHL were scares in the literature. However, our results are inconsistent with previous studies stated that 3 instances of patients with integrated HHV-6 into PBMC DNA have been illustrated, the results showed that the HHV6− IgM titer was negative and the IgG titer was either negative or at borderline level in each of three cases. [29,40]. Additionally, the present results are also in concordant with the study of [23], who assessed the HHV-6 IgM among lymphoma/myeloma patients; they were unable to detect IgM antibody among any serum sample.

The current results revealed that the HHV-6 DNA was undetected in 39 patients with NHL by PCR. Nevertheless, the possibility that the HHV-6 might be involved in the genesis of some B cell tumors began with the first hint that the HHV6 sequences associated with B cell tumors in a limited number of cases [41], who found that the viral sequence was detected in 4 out of 40 patients with NHL. Similarly results were reported by another study in the same year in which only 2 out of 117 patients with NHL were positive for HHV-6 DNA as detected by blot hybridization [42]. Furthermore, two other studies reported a total of three NHL patients were positive for HHV-6 DNA out of 113 patients examined, suggesting that HHV-6 is not likely to have a big etiologic effect in the developing of B-cell NHL [43,44]. Moreover, our results are also inconsistent with the study by [45] on 76 patients with NHL; he found that 59% of patients have detectable HHV-6 DNA in biopsy specimens by PCR assay.

Undoubtedly, when we talking about the detection of viral DNA, the type of specimen included and the type of detection tool are critical, and both are largely determine the study outcomes. For instance, application of conventional PCR in peripheral mononuclear cells of children patients with NHL, 33% and 10% of patients and control had detectable HHV-6

DNA respectively [38]. While utilization of immunohistochemistry and Southern blot techniques was failed to detect HHV6 DNA in lymph node biopsy samples [45,46]. Likewise, 45% of NHL patients were HHV-6 DNA positive by conventional PCR in formalin-fixed and paraffin-embedded lymph node tissues [18]. Similarly, 27 % of patients with different types of NHL were positive for HHV-6 DNA by PCR [47].

The presence of co-viral infection may also affect the detection rate. Since accumulated evidences on the role of certain viruses in causing NHL had been previously documented [48,49]. In this context, in a study on detection of herpes virus DNA (HHV6 or CMV) in patients with HL or NHL, the results showed that 46% were DNA positive in sample of WBC's or plasma by means of PCR assay and the same study found that 56% CMV infection were clustered among NHL cases [30]. HHV-6 DNA was identified in lymph node specimens of 53% from patients with AIDS-associated NHL versus 35% in HIV-seronegative patients with NHL [50] It is important to mention that a quantitative real-time PCR for detection of HHV6 A and B genome in plasma or blood samples (DIA-PRO, Diagnotic Bioprobes, Melano-Italy) was employed in our study which is highly sensitive and precise assay. However, the inconsistency of our results with others is largely attributed to the use of plasma instead of whole blood specimens. These findings revealed that there was an increased positivity rate of anti HHV-6 among patients with HL and NHL; however, the pathogenic role of the virus in the development these malignancies was difficult to be ascertain.

Conclusion: Although a higher anti-HHV-6 antibodies positivity rate among patients with HL and NHL, the pathogenic role of the virus in the development of these malignancies was difficult to be ascertain.

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