

RESEARCH ARTICLE

Association of Viral Infections with Risk of Human Lymphomas, Egypt

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Abstract

Background: The aim of this study was to determine and evaluate the association of different viral infections, with hepatitis B and C viruses, Epstein-Barr virus, cytomegalovirus and human herpes virus-8 (HBV, HCV, EBV, CMV, HHV-8) with the risk of lymphomas (Hodgkin and non-Hodgkin) among Egyptian patients, and correlate with the histopathological staging and typing as well as the prevalence of combined infections. **Materials and Methods:** A total of 100 newly diagnosed lymphoma patients with 100 healthy age and sex matched normal controls were assayed for viral infection using enzyme linked immunosorbant assay (ELISA) followed by real time polymerase chain reaction (RT-PCR). **Results:** Our results showed a high statistical significant difference between cases and controls as regards clinical and laboratory findings ($P < 0.001$ and $= 0.003$). A high statistical difference was seen for the association of most viruses and lymphoma cases ($p < 0.001$) except for positive HBs Ag, positive CMV IgG and HHV-8 ($p = 0.37$, 0.70 and 1.0 respectively). No statistical significant difference was found between Hodgkin (HL) and non-Hodgkin (NHL) as regards viral prevalence except HCV antigen, 57.1% for HL and 26.5% for NHL ($p = 0.03$). Only, HBV DNA showed a high significant value among infiltrated bone marrow cases ($p = 0.003$) and finally, a high significant association of 2 combined viral infections with infiltrated bone marrow lymphoma cases ($p = 0.04$). **Conclusions:** Our results showed that infection with HBV, HCV, CMV and EBV were associated with increased risk of lymphoma among the Egyptian population. Detection of new associations between infectious agents and risk of cancer development will facilitate progress in elaboration of prophylactic measures, early diagnostic methods and, hopefully, novel therapy of malignant tumours.

Keywords: Lymphoma - viral associations - ELISA - RT-PCR - HBV/HCV - EBV - CMV

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Introduction

Lymphoma is a cancer of the lymphatic system which is a part of our immunity. It is characterized by the formation of solid tumors in the immune system. There are different types of lymphoma depending on the lymphatic system affected, mainly two main types Hodgkin's lymphoma (HL) and Non-Hodgkin's lymphoma (NHL) (Kate et al., 2012). Lymphoma is a heterogeneous group of clonal lympho-proliferative disorders with diverse clinical presentation, pathogenesis, and biological behavior (Han Van 2015).

The World Health Organization (WHO) classification of neoplasms of the hematopoietic and lymphoid tissues, published in 2001 (Jaffe et al., 2001) and its updates in 2008 (Swerdlow et al., 2008), a worldwide consensus on the diagnosis of these tumors, adopted for use by the pathologists, clinicians, and basic scientists. The major principle of these classifications is the recognition of distinct diseases according to a combination of morphology, immunophenotyping, genetic, molecular,

and clinical features.

The disease entities are stratified according to their cell lineage and, additionally, their derivation from precursor or mature lymphoid cells. These principles in the 2001 WHO classification were based on the Revised European-American Classification of Lymphoid Neoplasms (REAL) published by the International Lymphoma Study Group in 1994.

Thus, the WHO classification of 2008 refined the definitions of well-recognized diseases, identified new entities and variants, and incorporated new emerging concepts in the understanding of lymphoid neoplasms. It adopted consensus guidelines for the definition of some well-established diseases, including chronic lymphocytic leukemia (CLL), Waldenström macroglobulinemia (WM), and plasma cell neoplasms. In addition, the WHO/European Organization for Research and Treatment of Cancer consensus classification of cutaneous lymphomas was largely adopted, the issue of grading of follicular lymphoma was revisited and the definitions of some categories of T-cell lymphomas, including enteropathy-

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associated T-cell lymphoma, anaplastic large cell lymphoma (ALCL), and subcutaneous panniculitis-like T-cell lymphoma, have been refined (Campo et al., 2011).

Human viral cancers are pathobiological consequences of infection with viruses that evolved powerful mechanisms to persist and replicate through deregulation of host oncogenic pathways, conveying cancer hallmarks to the infected cell.

Natural barriers to viral oncogenesis are the immune response and the innate safeguard for human cancer, environmental and host co-factors such as immunosuppression, genetic predisposition or mutagens can each affect this delicate equilibrium and could accelerate the development of these cancers. The infectious nature and long incubation periods of human viral cancers provide unique windows of opportunity for prevention and clinical intervention, one of which is lymphoma (De Martel et al., 2012).

Human oncogenic viruses rely on persistence to disseminate and thus deploy powerful immune evasion programs to establish long-term infections. As part of their replication and immune evasion strategies, human oncoviruses have evolved powerful anti-apoptotic and proliferative programs that can directly induce cancer hallmarks in the infected cell (Moore and Chang, 2010).

Hepatitis C Virus (HCV) an RNA virus. Although globally famous, due to its hepatotropism and hepatitis development, yet, a lymphotropic virus due to its association in recent years with some forms of non-Hodgkin's lymphoma (NHL), especially B cell NHL (Sayed et al., 2014). As for hepatitis B virus, whether an association between hepatitis B virus (HBV) infection and B-cell non-Hodgkin's lymphoma (B-NHL) risk exists is under studies (Yi et al., 2014).

Human Herpes virus-8 (HHV-8) is a γ 2-herpes virus the causal agent of Kaposi sarcoma (KS), characterized by the proliferation of infected spindle cells of vascular and lymphatic endothelial origin, accompanied by intense angiogenesis with erythrocyte extravasation and inflammatory infiltration (Ganem 2010; Mesri et al., 2010).

Among Herpes viruses, Epstein-Barr virus (EBV), a ubiquitous herpes virus that infects 90% of humans by adulthood, it is linked to the development of various cancers, including nasopharyngeal carcinoma, gastric cancer, burkitt's lymphoma, non-Hodgkin's lymphoma (NHL), and Hodgkin's lymphoma (Chang et al., 2013; Coghill and Hildesheim, 2014).

Cytomegalovirus (CMV) another oncogenic virus, its replication after allogeneic hematopoietic stem cell transplantation (HSCT) is not an independent variable for reduced risk of relapsed patients with B cell lymphomas, but is associated with lower overall survival and increased non-relapse mortality. Lymphoma histotype remains the main variable associated with increased relapse risk after HSCT, as for patients with HL and aggressive NHL showing a higher relapse rate compared with those with follicular lymphoma and pre-transplantation disease status (Mariotti et al., 2014).

In this study we aimed to determine and evaluate the association of different viral infections, including

hepatitis B virus, hepatitis C virus, Epstein-Barr virus, cytomegalovirus and human herpes virus-8 (HBV, HCV, EBV, CMV, HHV-8) with the risk of lymphomas (Hodgkin and Non Hodgkin) among Egyptian population, and correlate it with the histo-pathological staging, typing as well as the prevalence of combined infection among lymphomas.

Materials and Methods

This study was carried out on 100 newly diagnosed lymphoma cases, who presented to the Medical Oncology Clinics, National Cancer Institute, Cairo University, during the period from February 2009 to March 2011 with 100 healthy age and sex matched controls were included in the study.

A Written informed consent was approved by the Institutional Review board (IRB) ethical committee of the NCI which follows the rules of Helsinki IRB was obtained from each patient before starting the data collection. For the sake of patient's privacy, they were given code numbers.

Morphological and Immunophenotypic Analysis:

Morphologic analysis of bone marrow (BM) smear preparations was carried out at the Hematology Unit of the Clinical Pathology Department at the National Cancer Institute, Cairo University, Egypt.

A bone marrow aspirate and biopsy is part of the routine diagnostic work-up of a patient with suspected lymphoma, the diagnosis of lymphoma is based on pathology report and the value of bone marrow trephine biopsy in the diagnosis and staging of lymphoma is well established (Thomas et al., 2002). The panel considers a marrow trephine biopsy is a must, and should be performed in every patient to assess staging and bone marrow infiltration.

Blood and marrow smears are morphologically examined using a May-Gru'nwald Giemsa or a Wright-Giemsa stain and haematoxylin and eosin stain (H&E) for biopsy specimens. Paraffin-embedded sections were then cut and stained by basic immunohistochemical staining panel using monoclonal antibodies against pan-leucocytic marker (CD45), B-cell (CD20), T-cell (CD3), Hodgkin/RS cell (CD30), histiocyte (CD68) and proliferation (Ki-67) antigen markers. Corresponding clinical records were also evaluated, and diagnosis was made according to WHO proposal (Jaffe et al., 2008).

Immunophenotyping of fresh bone marrow samples was performed for all cases using flow cytometric analysis (Partec III from DAKO cytometry) (on routine basis), on marrow lymphoid cells with a panel of monoclonal antibodies, purchased from DAKO (Denmark), including FITC and PE conjugated CD 20, 19, 45, 5, 10, 11c, 22, 79a and 7 for cases of B cell lymphoma and CD 2, 3, 4, 5 and 8 for cases of T cell lymphoma. Specific isotype controls for FITC and PE conjugated monoclonal antibodies were used. Results were expressed as percentage of cells showing positive expression.

Samples

Blood samples were collected from all patients. The

serum was separated and stored at -80°C prior to analysis of viral assay.

Serological marker testing

Serological testing for the desired viruses was done using Enzyme Linked Immunosorbent Automated Spectrophotometric Analysis (ELISA) by using standard commercially available enzyme immunoassays according to the manufacturer's instructions:

HBs Ag (Biokit, S.A.BARCELONA – SPAIN biolisa HBs Ag color), HCV-Ab, CMV Ig M – Ig G and EBV Ig M – Ig G (Adaltis Italia S.p.a EIAgen HCV Ab “v.4” kit, EIAgen Cytomegalovirus Ig M – Ig G kit ,and EIAgen Epstein Barr VCA Ig M – Ig G KIT).

Viral antigen detection by PCR

All samples were subjected to antigenic detection of mentioned viruses by Real-Time PCR (RT-PCR) using standard commercially available kits supplied by (QIAGEN, Hilden, Germany) according to manufacturer's instructions.

RNA Isolation and Complementary DNA Synthesis for RNA Viruses (HCV)

RNA extraction was carried out using the QIA-amp viral RNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The concentration and purity of RNA was measured at 260 & 280 & 230 nm using Nano Drop 2000/2000c Spectrophotometer (Thermo Scientific, USA). Ratio of A260/A280 = 1.8 - 2.1 and A260/A230 = 1.8 - 2.1 indicates highly pure RNA. Extracted RNA was reverse transcribed into Complementary DNA (c DNA) using TaqMan® Gold RNA reverse Transcription Kit (P/N N808-0233) (Applied Bio-system, USA), which contains Multiscribe Reverse Transcriptase for first strand cDNA synthesis and thermal stable Ampli Taq Gold polymerase for second strand cDNA synthesis and DNA amplification.

The One step RT-PCR reaction was carried out in a total volume of 25ul for each reaction of which 10ul of extracted RNA was used with 15ul of universal Taqman master mix containing forward, reverse primers, fluorogenic probes and multiscribe reverse transcriptase. Then detected according to the following thermal cycle protocol: 48°C for 30 min. Reverse Transcriptase Step, 95°C for 10 min. DNA polymerase activation, 95°C for 15 sec. Denaturation Step, 60°C for 1 min. PCR annealing and extension using.

DNA Isolation for DNA viruses (HBV, CMV, EBV, HHV-8)

DNA extraction was done using QIAmp® viral DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions, (cat. # 51304).

The PCR reaction for each virus was carried out as follows

HBV- EBV and CMV: The PCR reactions were carried out in a total volume of 50ul for each reaction, 20 ul of extracted DNA and 30ul of universal Taqman master mix with forward, reverse primers and the fluorogenic probes. Then using the following thermal cycle profile: 95°C for 10 min. Taq enzyme activation, 95°C for 15 sec. denaturation step in PCR 50 cycles, 60°C for 60 sec.

annealing and extension step.

HHV-8

A total volume of 25ul for each reaction, of which 10ul of extracted DNA and 15ul of universal Taqman master mix with forward, reverse primers and fluorogenic probes using the following thermal cycle protocol: 95°C for 15 min. Taq enzyme activation, 95°C for 10 sec. denaturation step in PCR 50 cycles, 60°C for 60 sec. annealing and extension step.

Commercially available Positive and negative controls were run along with the clinical samples. To confirm the suitability of real-time PCR reaction conditions, the kit provides reagents for an internal positive control including primers and probes were used for normalization (Applied Bio-systems). PCR reactions were performed on The Applied Bio-system Step One Plus Real- Time PCR Systems (Applied Bio-systems).

Table 1. Demographic, Clinical and Haematological Findings in Lymphoma Patients and Controls

| | Cases | | Controls | | P- value |
|----------------------------------|-------|--------------------|----------|--------------------|----------|
| | N | (%) | N | % | |
| Sex | | | | | |
| M | 58 | 58% | 59 | 59% | 0.89 |
| F | 42 | 42% | 41 | 41% | |
| Spleen | | | | | |
| Negative | 79 | 79% | 100 | 100% | <0.001* |
| Positive | 21 | 21% | 0 | 0% | |
| Liver | | | | | |
| Negative | 94 | 94% | 100 | 100% | 0.03* |
| Positive | 6 | 6% | 0 | 0% | |
| Hepatosplenomegaly | | | | | |
| Negative | 87 | 87% | 100 | 100% | <0.001* |
| Positive | 13 | 13% | 0 | 0% | |
| Localized LN | | | | | |
| Negative | 53 | 53% | 100 | 100% | <0.001* |
| Positive | 47 | 47% | 0 | 0% | |
| Generalized LN | | | | | |
| Negative | 72 | 72% | 100 | 100% | <0.001* |
| Positive | 28 | 28% | 0 | 0% | |
| Extra-nodal | | | | | |
| Negative | 79 | 79% | 100 | 100% | <0.001* |
| Positive | 21 | 21% | 0 | 0% | |
| | Mean | Standard Deviation | Mean | Standard Deviation | |
| Age | 49 | 14 | 47 | 16 | |
| ALT(U/L) | 54.96 | 4.8 | 28.63 | 12.97 | |
| AST(U/L) | 65.65 | 6.4 | 29.05 | 11.25 | |
| Bilirubin(mg/dl) | 1.3 | 0.9 | 0.8 | 0.2 | |
| WBCs (x 10 ⁹ /L) | 11.24 | 7.34 | 5.91 | 2.05 | |
| HB (gm/dL) | 10.93 | 2.51 | 13.55 | 0.99 | |
| Platelets (x 10 ⁹ /L) | 202 | 154 | 298 | 84 | |

*Significant; LN (Lymphadenopathy), ALT (Alanine Transaminase), AST (Aspartate Transaminase), WBC (White blood cells), HB (Hemoglobin).

Data Analysis: By a standard curve method

The threshold cycle data (Ct) and baselines were determined using auto settings. The detection level in each specimen is determined by locating its Ct on the standard curve.

Statistical Analysis

Data management and analysis were performed using the Statistical Analysis System (SPSS) software. The study of prevalence of the studied marker among the studied groups was done by using Fisher Exact test. Comparison of groups with respect to numerical variables was done using the Mann Whitney test.

All p-values were two sided. P-values ≤ 0.05 were considered significant. Sensitivity, specificity and diagnostic accuracy were the validity measures used for testing the studied parameter

Results

In this study we investigated the possible association of viral infection and susceptibility of lymphoma among Egyptian population. Hundred adult newly diagnosed lymphoma cases and 100 healthy age and sex matched controls were included in this study. Blood samples of both patients and controls were subjected to Enzyme Linked Immunosorbent Automated Spectrophotometric Analysis (ELISA) to test for targeted viruses followed by antigenic detection of mentioned viruses by Real-Time PCR (RT-PCR).

Table 1. Summarize the clinical and laboratory characteristics of the patients and controls. Their mean ages were 49 ± 14 for patients and 47 ± 16 for controls, males represented 58% of all patients and 42% were females with a male to female ratio Of 1.38: 1.

As regards the clinical presentation, hematological and

laboratory findings, a high statistical significant difference was found between patients and controls (p-value= <0.03 , <0.001 and $=0.003$ respectively).

As regards the association of viral infection and lymphoma, viral marker analysis revealed a statistical significant difference between patients and controls for positive HCV Abs (47% for patients and 3% for controls) (P-value <0.001), positive CMV Ig M (12% for patients and 0% for controls) (P-value <0.001) and positive EBV Ig G (100% for patients and 90% for controls) (P-value= 0.001) and a non-significant difference for positive HBV s Ag (4% for patients and 1% for controls) (P=0.37), positive CMV Ig G (84% for patients and 82% for controls) (P=0.70). While antigenic detection using PCR showed a statistical significant difference for all viruses among patients and controls, for HBV DNA Positive cases were (16% for patients and 1% for controls)(p-value <0.001), HCV RNA positive cases (31% for patients and 1% for controls)(p-value <0.001), CMV cases were (11% for patients and 0% for controls)(p=0.001), EBV positive cases (22% patients and 0% controls) (p <0.001) and a non-significant difference for HHV-8 (1% for cases and 0% for controls)(p=1.0). Table 2 and Figure 1 summarize the

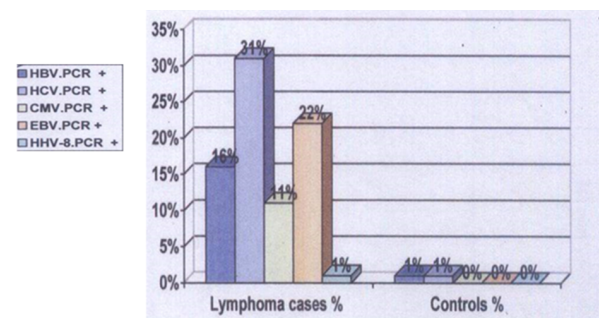


Figure 1. Viral Antigen Panel by RT-PCR

Table 2. Viral Markers Versus Antigenic Panel for Patients and Controls

| ELISA | | | | | PCR | | | | | | |
|----------|---------|------|---------|------|----------|----------|----|---------|-----|---------|----------|
| VIRUS | LYMHOMA | | CONTROL | | P-value | LYMPHOMA | | CONTROL | | P-value | |
| | Count | % | Count | % | | Count | % | Count | % | | |
| HBV | | | | | | | | | | | |
| Negative | 96 | 96% | 99 | 99% | P=0.37 | | 84 | 84% | 99 | 99% | P<0.001* |
| Positive | 4 | 4% | 1 | 1% | | | 16 | 16% | 1 | 1% | |
| HCV | | | | | | | | | | | |
| Negative | 53 | 53% | 97 | 97% | P<0.001* | | 69 | 69% | 99 | 99% | P<0.001* |
| Positive | 47 | 47% | 3 | 3% | | | 31 | 31% | 1 | 1% | |
| CMV | | | | | | | | | | | |
| IgM-ve | 88 | 88% | 100 | 100% | P<0.001* | Negative | 89 | 89% | 100 | 100% | p=0.001* |
| IgM+ve | 12 | 12% | 0 | 0% | | Positive | 11 | 11% | 0 | 0% | |
| Ig G-ve | 16 | 16% | 18 | 18% | P=0.7 | | | | | | |
| Ig G+ve | 84 | 84% | 82 | 82% | | | | | | | |
| EBV | | | | | | | | | | | |
| Ig G-ve | 0 | 0% | 10 | 10% | P=0.001* | Negative | 78 | 78% | 100 | 100% | P<0.001* |
| Ig G +ve | 100 | 100% | 90 | 90% | | Positive | 22 | 22% | 0 | 0% | |
| HHV-8 | | | | | | | | | | | |
| | | | | | | Negative | 99 | 99% | 100 | 100% | p=1.0 |
| | | | | | | Positive | 1 | 1% | 0 | 0% | |

*Significant

Table 3. Viral Prevalence among HD Versus NHL Cases

| | | HD | | NHL | |
|----------|-------|--------|-------|--------|---------|
| VIRUS | count | % | Count | % | p-value |
| HBV | | | | | |
| ELISA | | | | | |
| Negative | 14 | 100% | 79 | 95.00% | P=1 |
| Positive | 0 | 0% | 4 | 4.80% | |
| PCR | | | | | |
| Negative | 14 | 100% | 67 | 80.70% | p=0.12 |
| Positive | 0 | 0% | 16 | 19.30% | |
| HCV | | | | | |
| ELISA | | | | | |
| Negative | 10 | 71.40% | 42 | 50.60% | P=0.25 |
| Positive | 4 | 28.60% | 41 | 49.40% | |
| PCR | | | | | |
| Negative | 6 | 42.90% | 61 | 73.50% | P=0.03* |
| Positive | 8 | 57.10% | 22 | 26.50% | |
| EBV | | | | | |
| ELISA | | | | | |
| IgM-ve | 14 | 100% | 83 | 100% | p=1 |
| Ig G+ve | 14 | 100% | 83 | 100% | |
| PCR | | | | | |
| Negative | 11 | 78.60% | 65 | 78.30% | P=1 |
| Positive | 3 | 21.40% | 18 | 21.70% | |
| HHV-8 | | | | | |
| PCR | | | | | |
| Negative | 14 | 100% | 83 | 100% | P=1 |
| Positive | 0 | 0% | 0 | 0% | |
| CMV | | | | | |
| ELISA | | | | | |
| IgM-ve | 13 | 92.90% | 72 | 86.70% | p=1 |
| IgM +ve | 1 | 7.10% | 11 | 13.30% | |
| IgG-VE | 2 | 14.30% | 14 | 16.90% | |
| IgG +ve | 12 | 86% | 69 | 83.10% | |
| PCR | | | | | |
| Negative | 12 | 85.70% | 74 | 89.20% | p=0.66 |
| Positive | 2 | 14.30% | 9 | 10.80% | |
| Total | | | | | |
| ELISA | 14 | 100% | 83 | 100% | |
| PCR | 14 | 100% | 83 | 100% | |

*Significant

viral markers versus antigenic detection among patients and controls.

Among 100 lymphoma cases studied, 14% were HD and 83% were NHL. We assessed the association of different viruses with histopathology of the cases (HD/NHL) through serologic marker analysis versus antigenic detection.

Although most viruses were proved to be associated with malignant Lymphoma as mentioned before, yet, no statistically significant prevalence for the viruses among HD/NHL was found HBV (p=1 and 0.12), EBV (p=1), CMV (p=1 and 0.66), HHV-8 (p=1) except HCV antigen was higher among HD 57.1% than NHL 26.5% showing a significant difference (p = 0.03) Table 3.

Bone marrow infiltration was found in 36 lymphoma cases, association of viral infection among infiltrated and non-infiltrated cases showed no statistical significant difference (p>0.05) for all viruses shown in Table 4, except positive HBV DNA cases showed a highly significant value (30.6% infiltration versus 7.1% with no infiltration, p=0.003).

Table 4. Viral Association with Bone Marrow Infiltration

| | | Bone Marrow Infiltration | | | | P-value |
|-----------------|----|--------------------------|----|--------|------|---------|
| | | NO | | YES | | |
| VIRUS | | Count | % | Count | % | |
| HBV | | | | | | |
| HBs Ag Negative | 54 | 96.40% | 34 | 94.40% | 0.65 | 0.003* |
| HBs Ag Positive | 2 | 3.60% | 2 | 5.60% | | |
| PCR Negative | 52 | 92.90% | 25 | 69.40% | | |
| PCR- Positive | 4 | 7.10% | 11 | 30.60% | | |
| HCV | | | | | | |
| Ab Negative | 33 | 58.90% | 15 | 41.70% | 0.14 | |
| Ab Positive | 23 | 41.10% | 21 | 58.30% | | |
| PCR Negative | 37 | 66.10% | 27 | 75.00% | 0.36 | |
| PCR Positive | 19 | 33.90% | 9 | 25% | | |
| CMV | | | | | | |
| Ig M Negative | 50 | 89.30% | 30 | 83.30% | 0.41 | |
| Ig M Positive | 6 | 10.70% | 6 | 16.70% | | |
| Ig G Negative | 11 | 19.60% | 5 | 13.90% | 0.48 | |
| Ig G Positive | 45 | 80.40% | 31 | 86.10% | | |
| PCR Negative | 51 | 91.10% | 30 | 83.30% | 0.33 | |
| PCR Positive | 5 | 8.90% | 6 | 16.70% | | |
| EBV | | | | | | |
| Ig M Negative | 56 | 100% | 36 | 100% | 1 | 0.45 |
| Ig G Positive | 56 | 100% | 36 | 100% | | |
| PCR Negative | 43 | 76.80% | 30 | 83.30% | | |
| PCR Positive | 13 | 23.20% | 6 | 16.70% | | |
| HHV-8 | | | | | | |
| PCR Negative | 56 | 100% | 36 | 100% | | |

*Significant

Table 5. Combined Viral Infection by PCR and BM Infiltration (36 cases)

| PCR Combined | BM Infiltration | | Total |
|----------------------|-----------------|---------|---------|
| | No | Yes | |
| .00 count | 20 | 15 | 35 |
| % within combined | 57.10% | 42.90% | 100.00% |
| % within study group | 35.70% | 41.70% | 38.00% |
| 1.0 Count | 31 | 11 | 42 |
| % within combined | 73.80% | 26.20% | 100.00% |
| % within study group | 55.40% | 30.60% | 45.70% |
| 2.00 count | 5 | 9 | 14 |
| % within combined | 35.70% | 64.30% | 100.00% |
| % within study group | 8.90% | 25.00% | 15.20% |
| 3.00 count | 0 | 1 | 1 |
| % within combined | 0.00% | 100.00% | 100.00% |
| % within study group | 0.00% | 2.80% | 1.00% |
| Total Count | 56 | 36 | 92 |
| % within combined | 60.90% | 39.10% | 100.00% |
| % within study group | 100.00% | 100.00% | 100.00% |

*Significant P-value=0.04

Table 6. Frequency of Combined Viral Infection among Lymphoma Patients

| Combined viruses | Frequency | Percent |
|------------------|-----------|---------|
| HCV, EBV | 6 | 35.30% |
| HBV, HCV | 4 | 23.50% |
| CMV, EBV | 3 | 17.60% |
| HBV, CMV | 2 | 11.70% |
| HCV, CMV | 1 | 5.90% |
| CMV, HCV, HBV | 1 | 5.90% |
| Total | 17 | 100% |

Finally, we analyzed the association of combined viral infection among lymphoma cases with bone marrow infiltration. Our results showed that 25% of lymphoma patients with bone marrow infiltration had combined 2 viral infection compared with 8.9% without infiltration, and 2.8% of infiltrated cases had combined 3 infection compared to 0% without infiltration, showing a statistical significant difference ($p=0.04$). Table 5 and 6 Summarize the association of combined viral infection among lymphoma patients as regards bone marrow infiltration and frequency of combined viral infection.

Discussion

Malignant lymphoma represents a major health problem throughout the world. It is a common malignancy affecting B and T cell compartment of the immune system which is continuing to increase rapidly (Bakshi and Maghfoor, 2012). Many factors have been associated with the emergence of lymphoma one of which is viral infection, this plays a major role in involvement of malignant tumors (kutikhin et al., 2012).

We aimed in our study to detect a new association between infectious agents and risk of the development of cancer that might facilitate the progress in elaboration of prophylaxis measures, early diagnostic methods and, probably, methods of treatment of malignant tumors. The following viruses HVC, HBV, CMV, EBV, and HHV-8 were selected for the study due to their prevalence in Egypt.

In our study 100 newly diagnosed lymphoma cases after pathology confirmation, were 14 HL and 83 NHL cases with a mean age of 49 ± 14 and a male to female ratio of 1.38:1, this corresponds to a previous old study done by (Fisher and Fisher, 2004) and found that the median age at presentation for all subtypes of NHL in his patients was older than 50 years. Recently, (Dai et al., 2013), their study, "Incidence and trends of hematological malignancies in Japan and the United States", was in agreement with our results, they observed that the overall age-standardized incidence rates for all hematological malignancies per 100,000 in 2008 were 18.0 for males and 12.2 for females in Japan, and 34.9 for males and 23.6 for females in the US and that the most common type was the NHL particularly DLBCL, they stated that the incidence of a malignant disease in a certain population reflects the genetic and cumulative exposure to the environment of that population and evaluation of the incidence and secular trends of a disease in various populations may, therefore, be helpful in providing insights into the etiology and pathogenesis of that disease.

Our findings are consistent with those reported by (Saadettin et al., 2013), showing a similar if slightly higher mean male to female ratio but with a mean age difference than controls due to a larger sample size ($n=391$), they suggested that the overall HL incidence is increased during the second decade of life. However, the gender-specific age distribution of HL cases was different, while the distribution of female patients was similar to the overall distribution, in male patients the disease peaked twice in the 2nd and 4th decades of life.

In our study, positive correlation of hematological and laboratory findings as liver function tests and lymphoma were common and showed a high statistical significant difference from controls ($p\text{-value} = <0.003$ and <0.001). This is in consistent with (Polizzotto et al., 2015) as they correlated the clinical and laboratory features of their patients with Kaposi Sarcoma Herpes virus (KSHV) Inflammatory Cytokine Syndrome (KICS), and found that subjects compared with controls had more severe symptoms; lower hemoglobin and albumin and higher C-reactive protein. And, (Kathy et al., 2014) found that chronicity in a hematological patient (Hodgkin's Lymphoma, in their case) is common in immunosuppressed patients and it is important to monitor liver function tests and HEV viraemia. Chakraborty et al., 2015 also revealed severely impaired liver function test in case of lymphoma (Hodgkin) due to association of viral infection.

In our study, most viruses showed an association with lymphoma cases with a high statistical significant difference between patients and controls ($p \leq 0.001$) except in HBs Ag, a non-significant association was found with lymphoma cases ($p=0.37$).

Considering hepatitis B and C viruses, our results were in agreement with (Abe et al., 2015; Lindsay et al., 2014), both studies proved positive statistical association with HBV DNA, HCV abs and HCV antigen, the latter approved that both viruses increase risk for hepatocellular carcinoma, through both direct oncogenic effects and indirect effects, and have demonstrated increased non-Hodgkin lymphoma (NHL) risk with chronic HBV or HCV infection. However, the mechanisms by which chronic viral hepatitis may contribute to lymphomagenesis are unclear.

Nath et al., 2010 reached the same results of HBV correlation with lymphoma although a definite reason was not reached and further studies on the subject was encouraged. On the contrary to our results (Abe et al., 2015) found a high significant association of HBs Ag in his studied group with malignant lymphoma particularly NHL and diffuse large B-cell type which might be due to large population size studied ($n=20360$), so increasing our sample size and considering the differences in incidence in different places and among different populations would be important when investigating the aetiology of these diseases and help to detect higher significant values.

Egypt is considered to be one of the leading countries for HCV prevalence (Mahmoud et al., 2013). Our results showed a frequency of HCV abs in newly diagnosed lymphoma cases of 47% while HCV antigen was 31% by PCR with an agreement percent of 12%, those 35% who had HCV abs without RNA were presumed to have cleared infection, while the 19% who had evidence of HCV antigen by PCR but not antibody suggesting either acute infection or immunosuppression to old one thus suggesting that a chronic and not a transient infection with HCV has contributed to NHL. A similar conclusion has been reached by (Peveling-Oberhang et al., 2013) providing the 3 hypothesis in relation of HCV to Lymphoma correlation, one is continuous external stimulation of lymphoma cells by the virus, or HCV replication in B cells leading to oncogenesis and permanent B cell damage through (hit and

run) theory leading to tumor suppressor gene mutation.

As regards CMV, one of the eight members of the Herpesviridae family that commonly infect humans, and close to 100% of the adult population are infected with at least one of them (Grinde 2013). CMV is found all over the world among all socioeconomic standards, infecting between 50-85% of adults and associated with increased mortality after transplantation (Beam et al., 2015).

Our results showed a high significant difference between cases and controls as regards infection with CMV ($p<0.001$). Similar findings were detected by (Marchesi et al., 2015) who observed (11%) cases of lymphoma CMV positive individuals, Tyagi et al., 2015, also reached similar results of association in patients developing relapsed NHL after diagnosis of CMV retinitis confirmed by PCR.

EBV is a virus associated with rearrangement involving c-myc, combination with p53 protein and EBNA leading to B-cell immortality, it plays an important role in NK/T cell lymphoma and AIDS-associated lymphoma (Wang Y et al., 2015). EBV-DNA was detected by PCR in 22% of cases showing high statistical significance ($p<0.001$). On the same line, (Chang et al., 2013; Coghill and Hildesheim, 2014) found an association between antibodies against EBV proteins and the risk of EBV-associated malignancies. Immunoglobulin A antibody levels have been associated with the risk of nasopharyngeal carcinoma, and patients with Hodgkin lymphoma have significantly higher immunoglobulin G antibody levels than disease-free controls.

As for HHV-8, we found no association of lymphoma cases with HHV-8, no statistical significant difference was found ($p=1$), this was in accordance with a similar study done by (Nakamura et al., 2015) on both EBV and HHV-8.

In addition, Marianna et al., 2014 found that no blood donors were positive for HHV-8 antibody, thus reducing risk of transfusion related HHV-8 infection.

On the other hand, our results did not go in line with (Christenson et al., 2015) who found an association between human herpes virus 8 and primary effusion lymphoma, a rare subclass of non-Hodgkin's lymphoma principally seen in human immunodeficiency virus-positive patients, suggesting the presence of HIV co-infection. Similarly, (D'Antonio et al., 2011) in his study found HHV-8 combined EBV infection in lymphoma cells.

In this study, although 83% of patients were NHL and 14% were HL, and our results showed a strong association of viruses selected with lymphoma cases, yet, the differences among both groups (HL/NHL) as regards viral prevalence were not significant ($p>0.05$), except for HCV positive RNA were 57% among HL compared to 22% among NHL with ($P=0.03$). The lack of statistically significant difference among lymphoma subtypes may be attributable to the small sample size of this study, so increasing our sample size with more age variation may confirm or exclude such correlation. In agreement to our study, (Mwakigonja et al., 2008) found similar or slightly higher frequencies among his patients with 77% NHL and 22% HL except that he was reporting viral association with a different viral panel AIDS, and his results denoted a strong association with NHL. Similarly, (Corti et al.,

2010) worked on various viral panels & correlated it with histopathology, showing strong association between EBV and AIDS-related NHL and all cases studied were negative for HHV-8.

Finally, we analyzed the possible association of viral infection with progression of lymphoma as regards bone marrow infiltration, only HBV DNA positive cases showed a high statistical difference with 30.6% for infiltrated cases and 7.1% non-infiltrated ($p=0.003$). Our finding is in accordance with (Qingqing et al 2015) in his study, EBV associated T/NK-cell lymphoma, bone marrow involvement is 20-30% of cases and this is contributed to disease progression.

Our results are near to what was found by (Irsai et al., 2012), his study showed similar results of BM infiltration & multiorgan affection in lymphoma patients associated with viral infection especially CMV, he observed cytopathic effect in multiple organs and significant hemophagocytosis was also observed in the spleen, liver and bone marrow. In summary, active cytomegalovirus infection may be a major cause of multi-organ failure in the immunosuppressed patients.

On the other hand, we found a statistically significant association between bone marrow infiltration and combined viral infection, we found 25% of bone marrow infiltrated cases compared to 8.9% non-infiltrated cases had two combined viral infections ($p=0.04$), this is near to what was found by Ogawa et al., 2014 who found an association of multi organ infiltration with B cell lymphoma in presence of HHV-8 associated multicentric Castleman's disease in a patient with HIV infection, as well as, (Pinzone et al., 2015) who found EBV and Kaposi sarcoma-associated herpes virus-related malignancies in the setting of human immunodeficiency viral infection.

In conclusion, our preliminary results for the analysis suggested the association for EBV, HBV, CMV and HCV in the development of lymphoma among Egyptian population, while HHV-8 showed no significant correlation which might be contributed to the small sample size studied and its non-prevalence in Egypt, hence the difficulty in proving its correlation with the disease. Detection of new associations between infectious agents and risk of the development of cancer will facilitate progress in elaboration of prophylaxis measures, early diagnostic methods and, probably, methods of treatment of malignant tumors.

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