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# Differential expression of viral agents in lymphoma tissues of patients with ABC diffuse large B-cell lymphoma from high and low endemic infectious disease regions

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**Abstract.** Diffuse large B-cell lymphoma (DLBCL), the most common type of non-Hodgkin's lymphoma (NHL) in adults, accounts for approximately 30-40% of newly diagnosed lymphomas worldwide. Environmental factors, such as viruses and bacteria, may contribute to cancer development through chronic inflammation and the integration of oncogenes, and have previously been indicated in cervical cancer, hepatocellular carcinoma, gastric cancer and lymphoproliferative disorders. In the present study, the presence of microbial agents was analyzed in the lymphoma tissue of patients with activated B-cell like (ABC) DLBCL. The present study compared two groups of patients from geographically varied regions that possess a difference in the prevalence of viral and other microbial agents. The patient populations were from Sweden (a low endemic infectious disease region) and Egypt (a high endemic infectious disease region). A differential expression of several viruses in lymphoma tissues was noted when comparing Swedish and Egyptian patients. JC polyomavirus (JCV) was detected in Swedish and Egyptian patients and, uniquely, the complete hepatitis B virus (HBV) genome was detected only in Egyptian lymphoma patients. None of these viruses were detected in control lymph tissues from Sweden or Egypt. In total, 38% of the Egyptian patients were found to have HBV surface antigens (HBsAgs) in their serum; however, HBsAgs were not found in any of the Swedish patients. The percentage of serum HBsAgs in Egyptian patients with ABC DLBCL was significantly increased compared with the general Egyptian population (P<0.05). The present study may support a notion that viral agents, including JCV and HBV, may be involved in the tumorigenesis of DLBCL in regions of high infectious disease.

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## Introduction

Diffuse large B-cell lymphoma (DLBCL), the most common type of non-Hodgkin's lymphoma (NHL) in adults, accounts for approximately 30-40% of newly diagnosed lymphomas worldwide (1). Using gene expression profiling, DLBCL can be divided into three distinguishable subtypes; the germinal center B-cell like (GBC), activated B-cell like (ABC) and primary mediastinal B-cell lymphoma subtypes (1-3). These subtypes differ in the expression of thousands of genes and arise at separate stages of B-cell differentiation. The ABC subgroup has a poor prognosis with a short survival time compared with the GCB subgroup (2-4). In addition, the frequency of the ABC subtype in Asian countries, such as Japan and China, is increased compared with the GCB subtype. The ABC subtype is also more common in certain Asian countries compared with in the western world (5,6).

Environmental factors, including viruses and bacteria, may contribute to the development of cancer, and have been previously indicated in cervical cancer, hepatocellular carcinoma, gastric cancer and lymphoproliferative disorders (7-10). Viruses may be involved in the development of cancer by activating the innate immune system, as well as activating intracellular signaling cascades that control viral infection

and tumor cell growth (11). Bacteria, viruses and parasites may also directly contribute to carcinogenesis through the transfection and integration of oncogenes, which results in malignant cell transformation (12,13). Viruses involved in carcinogenesis may be targets for diagnosis, prevention and therapies (11).

Although the incidence of NHL has increased during the last three decades, the etiology of the most common types remains unclear (American Cancer Society, 2001). Chromosomal translocations are common in numerous NHL subtypes, which could result in the inactivation of tumor suppressor genes or activation of oncogenes (14). Viruses may induce genetic damage to the genome of lymphoid cells and dysregulate normal cell growth (15). Certain viruses have been specifically linked to particular types of lymphomas, including Epstein-Barr virus (EBV), human T-lymphotropic virus type 1, herpes virus-8 and hepatitis (16-18). Prevention of viral infections should reduce the number of individuals at risk of lymphoma development; thus recognizing the viruses involved in the tumorigenesis of NHL is important.

The virus genome has been shaped to interact with host cell regulatory and signaling networks. Viruses may alter host cell proteins in all steps of the viral cycle and utilize common host cell response pathways for pathogenic invasion (19,20). The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway may be activated by viral infections that result in maintained cell growth (21-23). Replicating hepatitis B virus (HBV) modulates STAT signaling by the upregulation of STAT3, which contributes to immune suppression, viral persistence and malignant transformation (21). As a part of the JAK/STAT pathway, STAT3 has been shown to be upregulated in several types of malignancies, including B-cell lymphomas (24). STAT3 has been previously suggested as a therapeutic target of ABC DLBCL, since STAT3 and nuclear factor κ B activation may be associated with a poor survival in this subgroup of patients (24).

A previous study analyzed the gene and micro RNA (miRNA) expression patterns in the lymphoma tissues of patients from two geographically varied regions with differing loads of microbial infections, and found a difference in the expression of STAT3 (25). The patient populations were from Sweden (a low infectious disease region) and Egypt (a high endemic infectious disease region) (25). STAT3 was overexpressed in Swedish patients compared with Egyptian patients (26) and miR-1234 was shown to be a possible regulator of STAT3 expression (27).

The main aim of the present study was to analyze the presence of microbial agents in the lymphoma tissues of patients with ABC DLBCL, and to associate this with STAT3 and miR-1234 expression.

## Materials and methods

*Patients and controls*. In total, 47 ABC DLBCL patient biopsy samples were obtained from the National Cancer Institute, Cairo, Egypt, between July 2003 and January 2008, and 14 ABC DLBCL patient biopsy samples were obtained from the Karolinska University Hospital, Stockholm, Sweden, between October 2005 and September 2011. The biopsy material was obtained at the time of diagnosis and embedded in paraffin. The median ages of the Swedish and Egyptian patients were 57 and 66 years, respectively. The gender distribution (% male/female) was 53/47 and 43/57 in Swedish and Egyptian patients, respectively. Reactive lymph nodes from teh same patients were collected as controls, from the National Cancer Institute, Cairo, Egypt (n=10) and from the Karolinska University Hospital, Stockholm, Sweden (n=10). The study was approved by the Regional Ethics Committee (www.epn.se) and written informed consent was obtained from all patients.

*Histopathological diagnosis*. Primary selection of DLBCL patients was performed using formalin-fixed (FF) paraffin-embedded (PE) material stained with eosin and hematoxylin and automated immunohistochemistry (IHC) for cluster of differentiation (CD)20, CD3, Ki67, B-cell lymphoma (Bcl)-2, Bcl-6, CD10 and melanoma associated antigen (mutated) 1, according to the algorithm described by Hans *et al* (28). This algorithm has recently been confirmed as one of two IHC algorithms that can predict the non-germinal center cell origin with a high accuracy, and has 86% concordance with gene expression profiling results (29). FFPE blocks classified as the non-GCB DLBCL subtype, as described previously (30), were selected for the study and were analyzed by the Department of Pathology.

*Handling of patient and control material*. The tissue blocks were cut into 20-$\mu$m thick sections, using sterile blades. All benches, instruments and pipettes were cleaned with RNaseZap solution (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A previous study reported that FFPE materials may be used for gene expression analysis, and are comparable with fresh frozen material (30).

*RNA extraction*. Extraction of RNA from PE material with the Ambion Recover All Total Nucleic Acid Isolation optimized for FFPE samples (Thermo Fisher Scientific, Inc.) was successfully performed following the manufacturer's instructions. RNA samples were stored at -80˚C until required for amplification. In brief, 20-$\mu$m thick sections were treated with xylene, centrifuged at full speed (10,000 x g) and washed with 100% ethanol twice. The pellets were air dried for 15 min. Digestion buffer and protease were added, followed by 3 h incubation at 50˚C. Isolation additive was added followed by 100% ethanol, and the samples were transferred to a filter cartridge. Two rounds of centrifugation with Wash 1 and Wash 2/3 was performed. DNase combined with 10X DNase buffer was added followed by 30 min incubation at room temperature. The membranes were then washed with Wash 1 and Wash 2/3. The RNA was eluted in 30 $\mu$l RNase free water preheated at 95˚C for 30 min. Yield and quality was measured using the Nanodrop Technologies ND 1000 (Thermo Fisher Scientific, Inc.). Absorbance ratios (260/280 for DNA and 260/230 for RNA) of >1.8 were considered to be of high purity.

*Complementary DNA (cDNA) preparation and amplification from PE DLBCL material*. RNA (100 ng) was used for amplification according to the protocols of the WT-Ovation FFPE RNA Amplification System V2 (Nugen Technologies, Inc., San Carlos, CA, USA), with a Spike control. All incubations were performed using the 1294 Techne Progene Peltier Thermal cycler thermal cycler (Scientific support, Inc.,

Hayward, CA, USA). The purification step was performed using Agencourt RNA Clean purification beads (Beckman Coulter, Inc., Fullerton, CA, USA). The amplified cDNA was purified using DNA Clean & Concentrator kit (Zymo Research Corporation, Irvine, CA, USA). The yield and quality of the cDNA was measured using the Nanodrop Technologies ND 1000. cDNA to be used in the microarray analysis were pooled using the same concentration of each sample.

*Microbial detection array (MDA).* Nucleic acid concentrations were determined using the Invitrogen Qubit Fluorometer (Thermo Fisher Scientific, Inc.). DNA and RNA were randomly amplified using a protocol described previously (31). Following amplification, the amplified samples were purified using the Qiagen QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany). Amplified nucleic acids were fluorescently labeled using the Roche NimbleGen One-Color DNA Labeling kit (Roche Applied Science, Madison, WI, USA), according to the recommended protocols. DNA was purified subsequent to labeling, and hybridized using the NimbleGen Hybridization kit (Roche Applied Science) and the Lawrence Livermore Microbial Detection Array (LLMDA), according to manufacturers' instructions. Microarrays were allowed to hybridize for 17 h and washed using the NimbleGen Wash Buffer kit (Roche Applied Science), according to manufacturer's instructions. The samples were then scanned on an Axon GenePix 4000B 5 $\mu$M scanner from Molecular Devices, LLC (Sunnyvale, CA, USA). Signals on the LLMDA were analyzed using the maximum likelihood analysis method (32). In brief, probes were identified when binding intensity to the probes exceeded a threshold equal to the 99th percentile of intensities for the negative control probes. Targets in an internal database of viral sequences were screened against stringency criteria and a log-odd score was computed for each target. A selection algorithm was applied to find the collection of targets most likely to be present in the sample. At every forward selection step, a conditional log-odd score was computed for each remaining target. The target with the largest conditional log-odd score was selected and added to the collection. The conditional log-odd score is a value representing the probability of being the detected target. Higher log-odd scores have an increased probability of being the detected target compared with lower log-odd scores.

*HBV antigen and antibody analyses of patient serum.* DNA extracted from the serum of Swedish and Egyptian patients was used to analyze the presence of the HBVsAg by polymerase chain reaction (PCR), by detecting HBV core genes as previously described (33). Briefly, 100 $\mu$l of reaction mixture containing 10 $\mu$l of extracted DNA, 50 mM potassium chloride, 10 mM Tris-hydrochloric acid (pH 8.3), 2 mM magnesium chloride, 200 $\mu$M deoxyribonucleosides, 2.5 units *Taq* polymerase (PerkinElmer, Inc., Waltham, MA, USA), and 20 pmol of each of the oligonucleotide primers [C1 sense, CTGGGAGGAGTTGGGGGA (1730-1747) and C2 antisense, GTAGAAGAATAAAGCCC (2503-2487)] for the core genes. Amplification was performed for one cycle of 95˚C for 5 min, followed by 35 cycles that consisted of denaturating for 1 min at 94˚C, annealing for 1 min at 55˚C and extension for 1.5 min at 72˚C. Antibodies against HBV were analyzed in Swedish and Egyptian patient serum using a chemiluminescent microparticle immunoassay (Abbott Diagnostics, Lake Forest, IL, USA). In brief, serum sample was mixed with paramagnetic microparticles coated with recombinant HBsAg (rHBsAg; Abbott Diagnostics). Subsequent to washing, acridium-marked rHBsAg-conjugate (Abbott Diagnostics) was added. Following additional washing, pre-trigger and trigger solution were added. The chemiluminescence reaction was then measured and the concentration of anti-HBs was set by a pre-designed calibration curve (Abbott Diagnostics).

*Relative expression of STAT3 in HBVsAg-positive (HBV+) and -negative (HBV-) Egyptian patients.* To evaluate the relative expression of STAT3 and micro RNA (miR)-1234 in association with HBV, HBV+ and HBV- Egyptian and Swedish patients were analyzed individually using Applied Biosystems HT7900 quantitative PCR (Thermo Fisher Scientific, Inc.). For the STAT3 relative expression analysis, the High Capacity RNA to cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for generating cDNA, according to manufacturer's recommendations. cDNA was further amplified (250 ng) by applying the TaqMan PreAmp Master Mix kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to manufacturer's recommendations. The TaqMan assays designed for STAT3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression of individual patient samples was calculated using the reference gene GAPDH. For the miR-1234 relative expression analysis, total RNA was poly-adenylated using yeast polyA polymerase (Affymetrix, Inc., Santa Clara, CA, USA) for 1 h at 37˚C, followed by 10 min at 65˚C, according to manufacturer's recommendations. The RNA was precipitated over night using sodium acetate-isopropanol at -20˚C, washed in ethanol and diluted in $H_2O$. The reverse transcription reaction was performed using the TaqMan miRNA reverse transcription kit (Thermo Fisher Scientific, Inc.) using 1.17 $\mu$M universal RT primer. The reaction was incubated at 72˚C for 5 min and put on ice prior to adding the RT enzyme. The reaction was then incubated at 42˚C for 45 min, followed by 5 min at 85˚C. qPCR was performed using an Applied Biosystems HT7900 qPCR machine (Thermo Fisher Scientific, Inc.) with miR-1234 primers and TaqMan universal PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression of miR-1234 was calculated relative to the expression of 5S rRNA. The mean Cq values and the relative expression for STAT3 and miR-1234 expression were calculated, as previously described (34).

*Statistics.* Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical calculation was performed by a one-way analysis of variance, followed by post-hoc Tukey's honest significant difference test or Student's t-test. $P<0.05$ was used to indicate a statistically significant difference.

## Results

*Microbial detection in Swedish and Egyptian patients using MDA analysis.* Viruses were detected by the MDA, a high-density

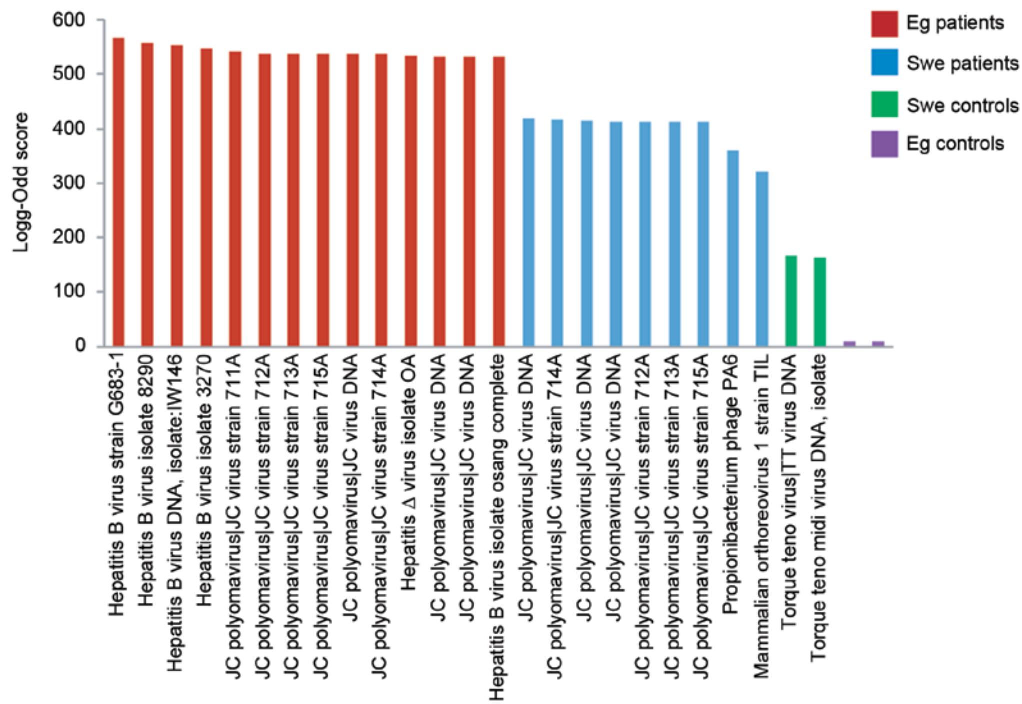


Figure 1. Bar graph showing detected viruses from the microbial detection array. Log-odd scores are indicated on the y-axis. Red bars indicate Egyptian patients, blue bars indicate Swedish patients, purple bars indicate Egyptian control samples and green bars indicate Swedish control samples. Each bar indicates the detection of a virus, including the complete genome, strain or isolate. Strains and isolate numbers are indicated. Eg, Egyptian; Swe, Swedish.

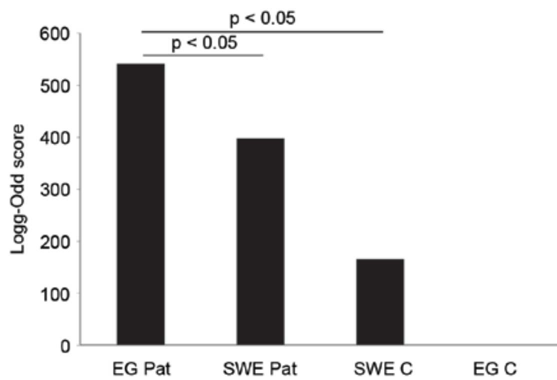


Figure 2. Bar graph summarizing log-odd scores for each patient or control group. Statistical analysis (Student's *t*-test) was performed and indicates statistical difference between the groups. No significant difference was shown between the controls sample groups. EG, Egyptian; SWE, swedish; C, control; Pat, patient.

oligonucleotide array for the detection and discovery of viruses and bacteria. Viruses that were found in all groups (controls and patients) were excluded, leaving exclusively those found in each patient group. Among excluded viruses were influenza virus A and human endogenous retroviruses found in the patient and control groups. Human immunodeficiency virus (HIV) was excluded as it was found in Swedish patients and controls. Clinically irrelevant viruses, including plant and animal viruses that have not been reported to infect human cells (for example faba bean necrotic yellow virus, porcine circovirus and melon necrotic spot virus) were also excluded. The log-odd scores were plotted in a bar graph (Fig. 1). The HBV had the highest log-odd scores, including the complete genome, and was only found in Egyptian patients. JC polyomavirus (JCV) was found in Egyptian and Swedish patients, with a higher log-odd score in the Egyptian patients, but not in Swedish and Egyptian controls. Generally, the highest log-odd scores were found in Egyptian patients rather than in Swedish patients. Few viruses were found in the control groups. To visualize the differences in log-odd scores between the groups, a mean log-odd score was calculated for each group. A statistically significant difference was found between the mean log-odd scores for each patient and control group (P=0.032 for Swedish controls versus Egyptian patients; P=0.041 for Swedish patients versus Egyptian patients; Fig. 2).

*Serum HBVsAgs and antibody responses.* HBV showed the highest log-odd score and the presence of the complete genome, but only in Egyptian patients using lymphoma tissues. HBVsAgs could be detected in the serum of 38% of Egyptian patients, but not in any of the Swedish patients. According to previous literature, the prevalence in the general population of Egypt for the HBsAg is 4% (35), and in Swedish patients <1% (36). The difference, with regard to the presence of HBVsAg, between the Egyptian patients and the general Egyptian population was statistically significant (P<0.05) (35). HBV antibodies could be detected in 100% of the Egyptian patients, but in none of the Swedish patients.

*STAT3 and miR-1234 in association with serum HBVsAg positivity.* Based on the presence of the HBVsAgs in serum, Egyptian patients were divided into a HBV+ and HBV- groups. All Swedish patients were HBV-. The relative expression of STAT3 did not differ between HBV+ and HBV-Egyptian patients, but was significantly increased in the Swedish patients compared with the total Egyptian patient population (P=0.00098 versus HB+ and P=0.00093 versus HB- Egyptian patients; Fig. 3A). A study has recently shown that miR-1234 may regulate STAT3,

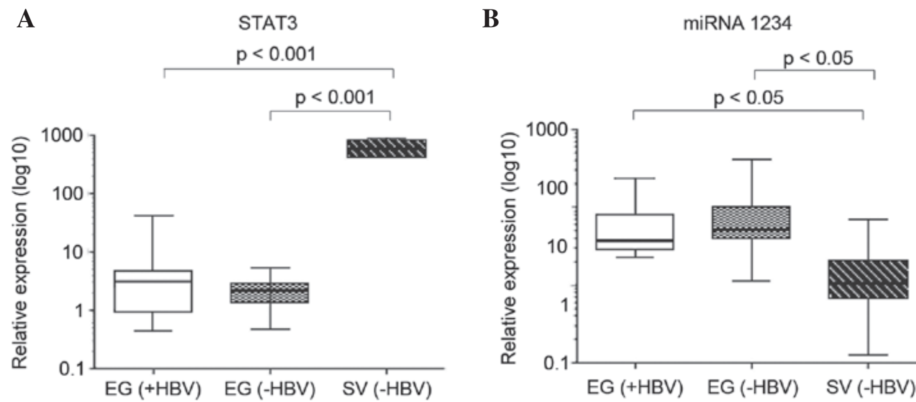


Figure 3. Box plot showing relative expression of (A) STAT3 and (B) miRNA in EG (+HBV) and EG (-HBV) patients and SV (-HBV) patients. Statistical analysis (analysis of variance) shows a significant difference between SV and EG patients. There were no statistical differences between EG (+HBV) and EG (-HBV) patients. EG, Egyptian, SV, Swedish; HBV, hepatitis B virus; STAT3, signal transducers and activators of transcription 3; miRNA, micro RNA.

and that the relative expression of miR-1234 is associated with HBV status (data to be published). In the present study, there was no difference in the relative expression of miR-1234 when comparing HBV+ and HBV- Egyptian patients; however, HBV- Swedish patients showed a significantly lower relative expression of miR-1234 compared with the total Egyptian patient population (P=0.044 versus HB+ and P=0.042 versus HB- Egyptian patients; Fig. 3B). miR-1234 expression was inversely associated with the expression patterns of STAT3.

## Discussion

Virus-linked human cancers may be responsible for 15% of all cancers, which makes viruses the second most important risk factor for cancer development after tobacco (37,38). The present study compared the presence of microbial agents in the lymphoma tissues of two groups of ABC DLBCL patients from a high (Egypt) and low (Sweden) infectious disease region (39). The LLMDA system used in the present study was developed at Lawrence Livermore National Laboratory, Livermore, CA, USA, and contains probes to detect any RNA and DNA of sequenced viruses and bacteria (32,40). Several viruses were found and most the prevalent were HBV and JCV. HBV was not detected in the Swedish patients or in either control group. JCV was also present in Egyptian and Swedish patients, but not in the controls. Overall, the Egyptian group showed higher virus log-odd scores compared with Swedish patients. The HBVsAg could be found in 38% of the Egyptian patients. There was no difference in STAT3 expression compared between the HBV+ and HBV- groups of Egyptian patients. STAT3 has previously been reported to be dysregulated by HBV, resulting in viral persistence and malignant transformation (21,41).

Viral infection of host cells may induce cell proliferation and immune suppression, which are mechanisms that may contribute to the viral-induced development of human tumors (21). HIV infected patients have an increased risk of developing, for example, Bcl and Kaposi sarcoma (38). Adenovirus and poxvirus have cell-transforming properties, but none of these have been found in human tumor cells (38). EBV has been recognized as a viral agent involved in the development of certain Bcls. Barzon *et al* (42) reported that patients with primary adrenal Bcl harbored EBV and JCV together, and suggested that these viruses may act as cofactors in the development of lymphomas (42).

Hepatitis B and C have been associated with hepatocellular carcinoma and dermatological, hematological, endocrinological and autoimmune disorders (43-45). HBV reactivation may be observed in patients during treatment with rituximab (46); however, no conclusive evidence of an association between NHL and hepatitis virus infection has been reported (47). Two studies showed that HBV infection was associated with an increased risk of DLBCL in Korean populations (48,49). An association between HBV infection and NHL was suggested in patients with persistent HBsAg (50). A study of a Korean population reported that 12% of NHL patients were HBV+ compared with 6% of the control population (51). According to the Centers for Disease Control and Prevention, 2-7% of the Egyptian population are HBV+ (52). These numbers were confirmed by a study reporting that 4% of Egyptian blood donors were HBV+ (35). The results of the present study show that lymphoma tissues of the Egyptian patients were HBV+ and that 38% of the patients were HBV+. None of the Swedish patients or any of the controls were HBV+. The results indicate that Egyptian patients have an increased viral load and that HBV was the most common virus. Since none of the Swedish patients were HBV+, the virus is unlikely to be associated with the development of DLBCL originating in Sweden. However, these results may be of importance for treatment strategies in Egyptian patients, since a previous study has demonstrated the reactivation of HBV in patients from high endemic infectious regions during treatment with rituximab (46).

The first human polyomaviruses (PyV), BK polyomavirus (BKV) and JCV were isolated in 1971 (53,54). Human PyVs are common in the general population but rarely cause clinically apparent disease (55). Sources of infection that have been suggested include virus-bearing fluids, contaminated food and water (56,57). The association of JCV with human malignancies is controversial; however, several studies have shown JCV to be associated with human cancers as colorectal-, gastric-, lung- and brain cancers (58-62). In a study by Flægstad *et al* (63), BKV was reported to be expressed in the tissue samples of neuroblastomas, but not found in control tissues. This suggests that BKV may be important in the development of neuroblastomas through the inactivation of cell cycle regulation and apoptotic effects, which contribute to malignant transformation (63).

BKV and JCV were recently classified as 'possibly carcinogenic to humans' by the WHO international cancer research working committee (64). The contribution of PyVs to cancer progression can be challenged in three ways First, the PyV may establish chromosomal instability at an early stage of infection that contributes to cancer progression, but PyV may not be detectable until full progression to malignancy. Secondly, PyVs may find favorable conditions in an already transformed cell, but be neither necessary for nor contribute to the oncogenic characteristics. Thirdly, PyV may be detected in an anatomically connected compartment, but be unrelated to the malignancy (55). JCV may establish a latent infection and e reactivated upon immunosuppression; one a site of latency has been shown to be lymphoid cells (65). The results of the present study showed that JCV may be detected in Swedish and Egyptian patients, but not in Swedish and Egyptian control groups. These data are notable as it may suggest a role for JCV in DLBCL.

Previous studies on Swedish and Egyptian patients showed that STAT3 was overexpressed in Swedish patients compared with Egyptian patients (26), and that miR-1234 may be a possible regulator of STAT3 (data to be published). No association between STAT3 and HBV expression was detected in the present study. However, the regulation of STAT3 by miR-1234 may also be associated with other environmental factors and to the increased viral load as noted in the Egyptian patients.

The current study showed that Egyptian and Swedish patients had detectable JCV, which was not detected in Swedish and Egyptian controls, and HBV was only detected in Egyptian patients. Extended studies on the viral involvement in DLBCL are warranted in order to analyze whether specific infections are associated with the development of the disease. Such studies may be of value for the development of novel prevention and treatment strategies.

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