Detection of Epstein Barr Virus Imp-1 Gene in Non Hodgkin Lymphoma Iraqi Patients

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Received 13 March 2014 Accepted 30 March 2014

Abstract
Non-Hodgkin's lymphomas (NHLs) are hematologic malignancy with the highest prevalence worldwide. They are broadly classified as B-cell or T-cell lymphoma depending on which type of lymphocyte becomes cancerous, B-cell lymphoma is more common than T-cell lymphoma. The EBV infects certain epithelial cells and linked to the development of multiple cancers, including NHL, HL, and nasopharyngeal carcinoma. This study designates to determine the frequency EBV lmp-1 in NHLIraqi patients, and to determine the correlation between EBV lmp-1 and subtypes of NHL. A 42 FFPE blocks were examined included 32 blocks from NHL patients, and 10 blocks from reactive follicular hyperplasia. Genomic DNA was extracted from these blocks using a specific kit and amplified by polymerase chain reaction (PCR) by using oligonucleotide primers specific for EBV lmp-1 gene. Our result shows EBV lmp-1 was detected in 43.8% cases of NHL, and in 10.0% cases of reactive follicular hyperplasia with no significant (P>0.05) variation between these groups. In addition to that, incidence of EBV lmp-1 was more frequently detected in high grade. From these results we conclude that EBV lmp-1 plays an important role in NHL development especially in high grade histopathological type.

Introduction
Lymphoma is a cancer of the lymphocytes, a type of white blood cells occurs when cells grow abnormally without of control [1].Traditionally, two main groups oflymphoma have been distinguished: Hodgkin Lymphoma (HL) characterized by large polynuclear cells called reed Sternberg cells, and Non-Hodgkin's lymphoma (NHL) [2]. Non-Hodgkin's lymphomas are the hematologic malignancy with the highest prevalence worldwide. They are broadly classified as B-cell or T-cell lymphoma depending on...
which type of lymphocyte becomes cancerous, B-cell lymphoma is more common than T-cell lymphoma. Researchers have found that NHL is linked with a number of risk factors, but the causes of most lymphomas are unknown. This is complicated by the fact that lymphomas are actually a diverse group of cancers [3]. Several viruses have been shown to play a role in the development of NHL like Epstein-Barr virus (EBV), Human T-lymphotropic virus (HTLV), and human immunodeficiency virus (HIV) [4]. Epstein–Barr virus or human herpesvirus 4 (HHV4) belongs to the genus Lymphocryptovirus within the subfamily of gammaherpesviruses. Common features of these viruses are their lymphotropism, their ability to establish latent infection of their host cells and to induce proliferation of the latently infected cells [5]. The EBV infects certain epithelial cells and linked to the development of multiple cancers, including Burkitt’s lymphoma, HL, some diffuse large B-cell lymphomas, and nasopharyngeal carcinoma [6]. The association between lymphoma and EBV came from the observation that EBV is the causative agent of infectious mononucleosis [7]. So that patients with a previous history of infectious mononucleosis have an elevated risk of developing lymphoma. The genome of EBV as in figure (1) consists of a linear, double-stranded DNA molecule that is 184 kilo base pairs in length. EBV has a series of 0.5 kb terminal direct repeats (TRs) and internal repeat sequences (IRs) that divide the genome into short and long, largely unique sequence domains [8].

Multiple EBV proteins can be expressed in infected lymphocytes, among which latent membrane protein-1 (LMP1) is thought to be most important for transformation [10]. Many molecular techniques are being used to demonstrate the presence of the EBV, such as the polymerase chain reaction (PCR) and in situ hybridization (IHS). The PCR makes it possible to detect minimal amount of viral DNA in tissues and smears [11]. This study was designates to determine the frequency EBV lmp-1 in NHL Iraqi patients, and to determine the correlation between EBV lmp-1 and subtypes of NHL paraffin embedded (FFPE) biopsy tissue blocks that were obtained from Iraqi patients and collected from the histopathology laboratories of Iraqi Hospitals and Private

**Subjects and Methods**

The subjects included in this study were represented as formalin fixed
Laboratories. Diagnosis of these tissue blocks were based on the obtained histopathological laboratory records of samples that had accompanied each tissue blocks in each laboratory. Also, a second histopathological reexamination of obtained tissue blocks was done by senior histopathologist. The collection samples of this study were carried out during the period from July 2012 until to April 2013. Study groups included 42 FFPE blocks. These samples were distributed as the following, 32 samples from patients of NHL, and 10 blocks from reactive follicular hyperplasia. The ages of NHL patients were ranged between 3-81 years with median age 55 years, and mean ± SD equal 45.718± 23.51 years. All NHL samples were taken before treatment and cases of NHL were classified according to the international working formulation (IWF ) of the National Cancer Institute to 3 groups [12] as table (1) elucidate.

Table (1): Non Hodgkin lymphoma subtypes enrolled in this study

<table>
<thead>
<tr>
<th>NHL subtypes</th>
<th>Number</th>
<th>Age (Mean ±SD) years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low grade</td>
<td>8</td>
<td>59.750±11.093</td>
</tr>
<tr>
<td>Intermediate grade</td>
<td>17</td>
<td>53.235±17.205</td>
</tr>
<tr>
<td>High grades</td>
<td>7</td>
<td>9.428±8.462</td>
</tr>
</tbody>
</table>

Formalin embedded blokes enrolled in this study sectioned by microtone, serial tissue sections were cut into 5-15 μm thickness from each tissue block. Genomic DNA was extracted from these sections by using Genomic DNA Minikit (Invitrogen Askutlanda) [13]. EBN lmp-1 gene was detected by using polymerase chain reaction. Amplification was performed in a programmable Multigene Thermal Cycler PCR (LabnetInternational Inc.USA). Primers sequences were revealed in table (2).

Table (2): Sequence of primers used for PCR amplification of EBV lmp-1 gene.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Size Product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'- ATTTATTTTGCTTGCCATT -3'</td>
<td>190 bp</td>
<td>14-15</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'- GTCTGTCTGTCTGTCCGTCA -3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PCR conditions
Genomic DNA was amplified in a final volume of 20 µl (5 µl Genomic DNA plus 3 µl forward primer plus 3 µl reverse primer plus 5 µl Bioneer’s master mix with Green Taq DNA polymerase plus 4 µl DDW) using the following conditions: Denaturation at 95 ºC for 5 min. followed by 35 cycles of (denaturation at 95 ºC for 30 seconds, annealing at 58 ºC for 30 seconds, and extension at 72 ºC for 1 min. and a final extension was at 72 ºC for 5 min. then hold at 4 ºC for indefinite time. Then the amplification products were separated by electrophoresis through 1.5% agarose gel (2%) stained with ethidium bromide (0.5 µg/ml).

Statistical Analysis
The data were analyzed using SPSS statistical software (SPSS version 16). P < 0.05 was considered statistically significant. The distribution and comparison of each was made using the Chi-square test. Odds ratio (OR) with 95% confidence intervals (CI) were estimated for the effect of high risk translocation.

Results
♦ Frequency of EBV *imp-1* Gene in Studied Groups
The EBV *imp-1* detected by conventional PCR in all tissues of studied groups. The present results illustrated positive results of EBV *imp-1* (43.8%) among 14 (32) NHL, while low percent was shown in reactive follicular hyperplasia 10.0% (1 out of 10) as shown in table (3). Statistically there were no significant differences (0.052) between studied groups. Otherwise, present results revealed that *imp-1* clinically consider as a risk factor in NHL development with risk estimate equal to 4 and (95%CI=0.654-29.264).

The outcome of amplification of DNA samples of EBV *imp-1* with selected forward and reverse primer was 190 base pair band which is our target, as in figure (2) illustrate.

Table (3): Frequency of EBV*imp-1* in studied groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Count</th>
<th>% within group</th>
<th>% within EBV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHL</td>
<td>18</td>
<td>56.2%</td>
<td>66.7%</td>
<td>100.0%</td>
</tr>
<tr>
<td>RFH</td>
<td>9</td>
<td>90.0%</td>
<td>10.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>% within EBV</th>
<th>33.3%</th>
<th>6.7%</th>
<th>23.8%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>Count</td>
<td>27</td>
<td>15</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>% within group</td>
<td>64.3%</td>
<td>35.7%</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>% within EBV</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

**Figure (2):** PCR amplification products of the EBV *lmp*-1 on ethidium bromide stained agarose gel (2%), 70 volt for 1 hr.

Lane (L): 100 bp DNA ladder

PCR products in the size region 190 bp are indicative of positive *lmp*-1.

A- Lane (3-5) positive of NHL.
B- Lane (1-3) positive of NHL, (7) positive of reactive follicular hyperplasia.

**Frequency of EBV*lmp*-1 in Non-Hodgkin Lymphomas Subtypes**

This study revealed that the relative incidence of EBV *lmp*-1 in high grade of NHL was (71.4%) higher than the intermediate and low grades that showed (41.2%, and 25.0%) respectively. Statistically there was no significant (0.186) variation within grades groups as in table (4). Also, our findings indicated...
that EBV lmp-1 is clinically more affected in high grades when compared with low and intermediate grades (CI 0.53-2.368) with odd ratio <1.

**Table (4):** Relationships between EBV lmp-1 and types of Non-Hodgkin's Lymphoma.

<table>
<thead>
<tr>
<th>Type</th>
<th>Count</th>
<th>N</th>
<th>P</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td></td>
<td>2</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Count</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within type</td>
<td></td>
<td>28.6%</td>
<td>71.4%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% within lmp-1</td>
<td>11.1%</td>
<td>35.7%</td>
<td>21.9%</td>
<td></td>
</tr>
<tr>
<td>Inter- medi- ate</td>
<td></td>
<td>10</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Count</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within type</td>
<td></td>
<td>58.8%</td>
<td>41.2%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% within lmp-1</td>
<td>55.6%</td>
<td>50.3%</td>
<td>53.1%</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>6</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within type</td>
<td></td>
<td>75.0%</td>
<td>25.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% within lmp-1</td>
<td>33.3%</td>
<td>14.3%</td>
<td>25.0%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>14</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within type</td>
<td></td>
<td>56.2%</td>
<td>43.8%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% within lmp-1</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
<td></td>
</tr>
</tbody>
</table>

$lmp-1$: Latent membrane protein-1, P: Positive , N: Negative

**Discussion**

- **Frequency of EBV lmp-1 Gene in Studied Groups**

Epstein Barr virus is a very common virus that is already infecting high percent of population worldwide and persisting for the lifetime in the host [16]. It is usually acquired in early childhood in developing countries. Despite the fact that EBV is a common infection, it has been postulated that EBV play a role in the pathogenesis of lymphoma [17]. Multiple EBV proteins can be expressed in infected lymphocytes, among which LMP1 is thought to be most important for transformation [10].

Overall, EBV lmp-1 detected in our study among all NHL was 43.8% (14 out of 32 cases), among reactive follicular hyperplasia was seen in 10% (1 out of 10 cases). Many research revealed that the EBV was associated with lymphoma [18-20]. Naturally EBV has a receptor on B lymphocytes called complement receptor (CR21 or CR2) which at the same time has mutagenic characteristics for B lymphocytes. In other words, it is the cause of the polyclonal stimulation of cells. Following the contamination of epithelial cells the active replication of the virus leads to the lysis and destruction of the cells [21-22]. Moreover, EBV causes the infected B cells to replicate and this leads to a genetic mutation in new B cells and eventually transformation to lymphomas [23-24]. Also, LMP-1 is expressed in many EBV-associated cancers and is responsible for most of the altered cellular growth properties that are induced by EBV infection [25-26]. The LMP-1 is functionally similar to CD40, acts as a constitutively activated receptor 9 and can activate NF-kB signaling and
downstream genes: the anti-apoptotic bfl-1 gene [27].
Numerous studies recorded different EBV positivity in NHL case, Goninet al. showed the positivity of EBV in 30% of NHL [28]. Others illustrated the EBV positivity was seen in 12.7% of (9 / 71) cases while the high EBV genomes were detected in 68% of all NHL [29]. Furthermore, Tumwine et al showed the frequency of expression of LMP-1 of EBV was detected in NHL patients (34.7%) [30]. In contrast with [31] that shows DNA EBV detected in (9/13) (69.2%) of reactive follicular hyperplasia, and low incidence in NHL (4.8%). When compared with previous results mentioned above, we can see many of them in agreement with our results.
Despite the frequency of EBV infection of NHLs is influenced by various factors such as the patient's immune status, the disease histologic subtype, the anatomical site of the tumor, and the sensitivity of the detection method. [32-33]. The current indicated EBV LMP1 clinically has a significant role in lymphoma development (95% CI=0.654-29.264) without any history of patients immunity that closely related to different studies were reported higher association of the EBV with NHL as high as 80% in other developing countries [34-35].

Frequency of EBV Lmp-1 in Non Hodgkin Lymphomas Subtypes

Epstein Barr virus is an important example of a transforming virus implicated in several NHL subtypes [36]. Non Hodgkin Lymphoma in present study was subdivided into three groups, high, intermediate, and low grades. High presence of Lmp-1 gene was detected in high grades (71.4%) of NHL patients. These results were in agreement with some previously studies which also found 30% EBV positive in their NHL cases [37-38]. It is also worth mentioning that Burkitt’s lymphoma is a high-grade malignant NHL that is most commonly associated with EBV infection [39-40]. In United States, EBV associated with Burkitt’s lymphoma documented in 20% of patients [16]. Previous study observed the carcinogenic role of EBV in Burkitt's lymphoma when find that EBV was a potent transforming virus in culture for the same cell type that develops into Burkitt's lymphoma [41]. Others demonstrated that Burkitt's lymphoma is highly associated with (EBV) in over 95% of cases while in Egypt and Brazil Burkitts lymphoma have also been documented up to 87% of tumors are EBV positive [42-43]. The roles of EBV contributes to the B-cell cancer pathogenesis by expressing EBV-encoded LMP1, as well as enhancing genetic instability through mutation, translocation, and dysregulated expression of proto-oncogenes [44]. Our results also reported low frequency of EBV in low and intermediate grades that indicated the less impact of these proteins in these types that consistence with [40]. Statistically insignificant correlation observed between EBV and subtypes of NHL, but from CI results we can predicate the clinical role of EBV in NHL subtypes especially with Burkitt's lymphoma.

The variable percentages in different studies may be due to the fact that EBV prevalence may be different in different parts of the world.

Conclusion

In conclusion the highest frequency of Lmp-1 gene in NHL in our study is 71.1% of high grade and is mostly seen in Burkitts lymphoma. This requires further evaluation to find out whether this positivity is due to co-infection or has a role in pathogenesis.

References


