Expression levels of caspase-3 in EBV positive and negative Hodgkin Lymphoma patients

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Abstract

Background: Many studies indicated that caspase-3 is overexpressed in Hodgkin Lymphoma. This study was aimed to investigate gene expression of caspase-3 in peripheral blood of EBV positive and negative HL patients. Methods: Blood samples were collected from two groups; the first group was 61 Hodgkin Lymphoma patients and the second group was 36 healthy individuals as a control group. Conventional PCR used to detect EBV in Hodgkin Lymphoma patients, RT-PCR used to evaluate expression of caspase-3. Results: Data from current study showed that 26 patients were EBV positive (42.6 %). Also, caspase-3 was expressed in HL significantly more than control group (P=0.001), Statistical analysis revealed that the difference in gene expression of caspase-3 between EBV positive and EBV negative cases, although high, was statistically insignificant (P= 0.942). Conclusion: Caspase-3 were over expressed in Hodgkin Lymphoma patients, however, caspase-3 was lower in EBV positive than EBV negative Hodgkin Lymphoma.

Keywords: Hodgkin Lymphoma, EB virus, caspase-3, PCR.

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Introduction

Hodgkin’s lymphoma (HL) is a lymphoid malignant tumor forming less than 1% of all de novo neoplasms occurring worldwide annually. Diagnosis of the disease is based on the identification of characteristic multinucleated giant cells within an inflammatory milieu. These cells are called Reed-Sternberg (RS) (¹). According to Iraqi cancer board, HL is one of the most ten common cancer in Iraq. 479 cases of HL were recorded in 2011 while in 2016, 517 cases were recorded with incidence rates of 1.54% and 1.34% in males and females, respectively (²)
The etiology of HL is largely unknown. However, higher risks have been reported in those with autoimmune diseases, persons with higher socioeconomic status, smaller families, those with congenital and acquired immunodeficiency, those with family history of HL or other lymphoid neoplasms and those with Epstein–Barr virus (EBV) latent infection. EBV is a ubiquitous virus that infects about 95% of the world population at some point in life. EBV Chronic infections and reactivation of latent infection are associated with many types of tumors including Hodgkin lymphoma.

Caspases are proteases that control intracellular propagating programmed cell death, proliferation and inflammation. Caspase activation occurs through a preserved mechanism subject to strict cellular control. Caspases included in apoptosis had been sub-classified according to their mechanisms of actions as initiator caspases (caspase-8 and -9) and executioner caspases (caspase-3, -6, and -7). Active caspase 3-positive neoplastic cells have been associated with the expression of p53 and its downstream effector molecule p21, suggesting that the stress-induced apoptosis pathway works properly. This study was aimed to investigate gene expression of caspase-3 in peripheral blood of EBV positive and negative HL patients.

Methods

Patients and sample collection

Present study was a case-control study based on two groups. The first group was 61 patients with Hodgkin lymphoma that clinically and histopathologically diagnosed and not received chemotherapy. Blood samples were collected from patients who attended the Department of Oncology at Al-Diwaniyah Teaching Hospital and Middle Euphrates Center for Tumors in Najaf city, Iraq, in the period from January to August 2018. Patients groups were sub-divided into EBV positive and EBV negative according to presence of LMP-1 gene. Data collected from patients included age and gender. The second group included 36 healthy volunteer individuals. Five millimeter of venous blood samples of venipuncture were collected from these groups by drawing using disposable syringe under aseptic technique. The blood was placed directly in a sterile tube containing EDTA for DNA and total RNA extraction. Conventional and real time PCR primers used in this study were designed by using NCBI-Gene Bank data base and Primer 3 design online.

Primers and probes

Conventional and real time PCR primers used in this study were designed by using NCBI-Gene Bank data base and Primer 3 design online. These primers and probe (Table 1) were provided by (Bioneer Company, Korea).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Product Size</th>
</tr>
</thead>
</table>

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DNA and RNA Extraction

Genomic DNA from blood samples was extracted by using Genomic DNA mini kit extraction kit (Frozen Blood) (Geneaid, USA), and done according to manufacturer's instructions. Total RNA was extracted from blood samples by using (TRIzol® reagent kit. Bioneer, Korea) and done according to company's instructions.

Conventional PCR

DNA templates of patients were subjected to PCR using LMP-1 primer (F and R). All PCR components were mixed on ice under sterile condition, assembling PCR materials were done according to the procedure of INTRON (Korea) mastermix kit using 20μl reaction volumes. PCR machine was installed for cycling according to primer condition as the following conditions: Initial denaturation 95/2min, denaturation 95/30sec, annealing 55/30sec, extension 72/1min and final extension 72/5 with 30 cycles. The PCR products were analyzed by agarose gel electrophoresis (1% agarose).

RT-PCR

RT-qPCR used to evaluate gene expression of caspase-3 and that normalized by housekeeping gene (GAPDH) in blood samples of HL patients and control group. The ∆∆CT method, also referred to as the Comparative CT method, is a means of measuring relative quantification and was described by Livak and Schmittgen 2001(9)

Statistical analysis
Data were summarized, presented and analyzed using statistical package for social science (SPSS version 23) and Microsoft office Excel 2016. Numeric data were presented as mean, standard deviation, range, median and Inter-Quartile Range (IQR) after performance of Kolmogorov-S normality test and making decision about normally and non-normally distributed variables. Data that were not normally distributed were transferred to Log form and Student’s t-test used to compare between two means. Chi-squared test was used to study association between any two categorical variables. Odd ratio and 95% confidence interval were estimated to measure risk. The level of significance was considered at \( P \)-value of 0.05 or less.

**Results**

**Demographic Characterization**

According to age, patients with Hodgkin’s lymphoma in this study were distributed as following: 5(8.20%), 35(57.38%), 15(24.59%) and 6(9.84%) as <20 years, 20-39 years, 40-59 years and \( \geq 60 \) years, respectively, with a median age of 35 years. Thus, the disease has been mostly encountered at young adulthood between 20-39 years age interval (Figure 1). The present study revealed that the mean±SD age of patients with HL was 36.51±15.23 years and that of control group was 38.15±16.58 years. The difference was statistically insignificant (\( P = 0.654 \)).

![Distribution of HL According to Age](image)

**Figure (1) Histogram showing the distribution of patients with Hodgkin’s lymphoma according to age.**

With respect to distribution of patients with HL according to gender, these results showed that there were 33 male patients and 28 female patients accounting for 54.10% and 45.90%, respectively, (Table 2). On the other hand, control group included 17 men and 19 women accounting for 47.2% and 52.8%, respectively, (Table 2). There was no significant difference in the distribution of patients and control
groups according to gender \( (P= 0.497; \text{Table 2})\). The distribution of the disease was found to be more common in males than females; Male to female ratio was 1.18:1.

Table (2) Frequency distribution of patients with Hodgkin’s lymphoma and control subjects according to gender

<table>
<thead>
<tr>
<th>Gender</th>
<th>Control ( n=26 )</th>
<th>Hodgkin’s lymphoma ( n=61 )</th>
<th>( \chi^2 )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, ( n (%) )</td>
<td>17(47.2%)</td>
<td>33(54.1%)</td>
<td>0.429</td>
<td>0.513 ¥ NS</td>
</tr>
<tr>
<td>Female, ( n (%) )</td>
<td>19(52.8%)</td>
<td>28(45.9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( n \): number of cases; ¥: Chi-squared test; NS: none significant at \( P\leq0.05 \).

According to residency, patients with Hodgkin’s lymphoma were categorized into 24(39.3%) and 37(60.7%) from Al-Diwaniyah and Al-Najaf provinces, respectively.

Detection of EBV

Conventional PCR conducted to detect \( EBV \) LMP-1 gene in extracted DNA from blood of HL patients. PCR results showed that 26(42.6%) patients, out of 61, were \( EBV \) positive. LMP-1 positive samples gave bands equal to target product size of the primer (531bp; Figure 2).

![Ethidium bromide-stained agarose gel of PCR products amplified with EBV LMP-1 gene HL patient extracted DNA (531bp). Lane (L) is a 100bp standard size reference marker. Lanes (1-10) are LMP-1 positive cases.](image)

Caspase-3 gene expression
Current investigation included evaluation of caspase-3 gene expression for HL patients and the control group using RT-PCR technique. The statistical analysis showed that there were highly significant differences \( (P=0.001) \) between gene expression of the two groups (Table 3).

### Table (3) Caspase-3 folds change value in patients with HL and control group.

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Controls</th>
<th>HL patients</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>22.26±76.692</td>
<td>146.09±446.00</td>
<td>0.001 † HS</td>
</tr>
<tr>
<td>Range</td>
<td>0.02-393.20</td>
<td>0.14 -656.21</td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>0.64(6.45)</td>
<td>4.19 (65.35)</td>
<td></td>
</tr>
<tr>
<td>( n )</td>
<td>36</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

*\( n \): number of cases; †: Independent sample t-test using log transformation; SD: standard deviation; IQR: inter-quartile range; HS: Highly significant at \( P \leq 0.01 \)*

Assessment of Caspase-3 gene expression was further compared between the two subgroups, EBV positive and EBV negative HL patients, and the results were shown in Table (4). Statistical analysis revealed that the difference in fold change between EBV positive and EBV negative cases, although high, was statistically insignificant \( (P= 0.942) \).

### Table (4) Caspase-3 fold change value in patients with Hodgkin’s lymphoma categorized into EBV negative and positive cases

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Negative</th>
<th>Positive</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>174.86±533.93</td>
<td>107.36±294.91</td>
<td>0.942 † NS</td>
</tr>
<tr>
<td>Range</td>
<td>0.14 – 656.21</td>
<td>0.82 -1103.62</td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>1.33 (12.85)</td>
<td>5.61 (64.44)</td>
<td></td>
</tr>
</tbody>
</table>

*\( n \): number of cases; †: Independent sample t-test; SD: standard deviation; IQR: inter-quartile range; NS: none significant at \( P \leq 0.05 \).*

### Discussion

The present study revealed that the mean±SD age of patients with HL was 36.51±15.23 years, this results disagreed with local study conducted in Erbil and showed that median is 23 \(^{(10)}\), however, this may be due to difference in sample sizes of the studies. Current data agreed with \(^{(11)}\). In the United States, HL is most often diagnosed at an age interval (20-34) years of age, with a median age of 38 years. However, patients at any age can get the disease \(^{(12)}\). HL epidemiology is distinguished by its variability in incidence at diagnosis by age. This is reflected by the well-known bimodal curve in industrialized countries with two peaks: the most important for young adults (15–34 years) and the second one in is later life (over 50 years) \(^{(13)}\).

Male to female ratio in this study was 1.18:1. These results agreed with Iraqi Cancer Board report in 2011 and 2016, in which male to female ratios were 1.2:1 and 1.17:1, respectively \(^{(14)}(2)\). HL, in males...
is more than females, was also reported by studies in different parts of the world \(^{(11,15-20)}\). In addition, current results disagreed with \(^{(21,22)}\). Epidemiological studies had shown that there were independent associations between age, gender, race, geography and HL incidence. The variability in incidence by age, place, social class, and time indicated an etiological role for infectious agents such as EBV while aggregation in families and individuals with specific human leukocyte antigen \(^{(23)}\).

Multiple studies around the world assessed the strong link between HL and EBV infection with several facts, for instance, elevated antibody titers of EBV in patients with HL who have had primary EBV infectious mononucleosis. The risk of developing HL is increased to threefold and the presence of EBV nucleic acid in HL tissues indicated that EBV infection may play a crucial role in the pathogenesis of HL as a primary event in the development of HL \(^{(24)}\).

Although the high expression of caspase-3 in HL, the caspase-3 does not fight tumor cells, this may by due to the presence of a group of molecules called inhibitors of apoptosis proteins (IAP) which can deactivate Caspases. Many members of IAP, like XIAP, cIAP1, and cIAP2 are able to inhibit the effector caspase-3 directly. Since cIAP2 was strongly expressed in HRS cells in majority of examined cHL cases, it could be important for silencing CD95-mediated pro-apoptotic signals and for the survival of HRS cells by blocking caspase-3 \(^{(25)}\).

Moreover, apoptosis can be regulated by other anti-apoptotic protein families such as bcl-2 and bax. Increased bcl-2 expression is a possible cause of apoptotic cascade blockade upstream of caspase-3, however, investigations showed that B cells lacking caspase-3 showed increased proliferation \textit{in vivo} and hyperproliferation after mitogenic stimulation \textit{in vitro}, this hyperproliferative B-cell phenotype was rescued in double-knockout mice lacking both caspase-3 and the cyclin-dependent kinase inhibitor p21 (encoded by \textit{Cdkn1a}), which is a caspase-3 substrate \(^{(7)}\).

Present investigation revealed that caspase-3 was upregulated in peripheral blood of HL patients. Over expression of caspase-3 was also reported in B cell lymphoma, including HL \(^{(26-29)}\).

Present results demonstrated that caspase-3 gene expression in EBV positive HL patients was lower than in EBV negative patients. These results indicated that \textit{EBV} could decrease the expression levels of caspase-3. However, some investigations tried to explain this downregulation. For example, \(^{(30)}\) demonstrated that EBV LMP2A increased the expression of genes associated with cell cycle induction and inhibition of apoptosis, altered the expression of genes involved in DNA and RNA metabolism, and decreased the expression of B-cell–specific factors and genes associated with immunity \(^{(30)}\).

Moreover, EBV infection is associated with the expression of COX-2, p16INK4A and p53 that regulate apoptosis and proliferation of tumor cells in HL \(^{(31)}\).

**Conclusion**

This study revealed that miRNA-155 expression in EBV- positive was more than EBV-negative while caspase-3 expression in EBV- positive was lower than EBV-negative in peripheral blood of HL patients.
Ethical Clearance

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest

The authors declare that they have no conflict of interest

Funding: Self-funding.

References


