Bcl-2 Expression in Patients with Non Hodgkin's Lymphoma with Hepatitis C

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Abstract

The mechanism of lymphoma genesis in hepatitis C virus-related, B cell NHL is unknown. It has been suggested that HCV may induce B cell clonal proliferation and BCL-2 gene overexpression in chronic HCV patients.

Aim of the Work: Is to evaluate the expression of BCL-2 gene in patients with NHL with HCV infection.

Methods: The study included 92 patients, divided into 52 patients with B-cell NHL with chronic HCV infection and 40 patients with B-cell NHL without HCV infection who attended the National Cancer Institute, Cairo University, during the period from March 2012 to May 2014. Lymph node biopsies from all cases were studied for Bcl-2 expression using tissue microarray and immunohistochemistry techniques.

Results: BCL-2 was expressed in 32.7% of cases with B-cell NHL with hepatitis C and in 20% of cases of HCV negative NHL by immunohistochemical method and the Bcl-2 gene was up-regulated more than 2 folds in NHL HCV+ve patients than in NHL HCV−ve group by tissue microarray technique with statistically significant difference.

Conclusion: Bcl-2 is over expressed in NHL with HCV infection more than in NHL without HCV and could play a role in lymphoma genesis and future therapeutic strategies.

Key Words: Bcl-2 – LN – Non Hodgkin lymphoma – Hepatitis C virus.

Introduction

INFECTION with HCV affects an estimated 180 million people accounting for 3% of the global population [1,2]. The Egyptian Demographic Health Survey (EDHS) estimated HCV prevalence among the (15-59) years age group to be 14.7% [3]. Accordingly, Egypt has the highest HCV prevalence in the world [4]. The association between HCV and NHL is strongest in geographical areas with highest prevalence of viral infection [5].

Non-Hodgkin's lymphoma is among the hematological malignancies with high prevalence worldwide constituting 5.1% of all cancer cases and 2.7% of all cancer deaths [6]. An estimated 355 900 new cases and 191 400 deaths have been attributed to NHL in 2008 [7]. Non-Hodgkin's lymphomas have a high rate of genetic alterations like translocations detectable in up to 90% of NHL [8]. Recently, many studies have proposed that different individual etiological mechanisms are not mutually exclusive, but development of NHL is a multi-causal event [8,9].

A causative relationship between HCV and NHL was postulated relatively recently and has been the subject of intense investigations as well as some debate. HCV appeared to be involved in the pathogenesis of at least a proportion of patients with NHL subtypes including marginal zone lymphoma (splenic, nodal and extranodal), small lymphocytic lymphoma/chronic lymphocytic leukemia, lymphoplasmacytic lymphoma and diffuse large B-cell lymphoma. Remarkably, some HCV-associated NHL appeared to be highly responsive to antiviral therapy, providing some clinical evidence for the causal relationship as well as the prospect for new therapeutic intervention [10]. HCV sequence has been detected in lymph node biopsy specimens from patients with B-NHL and the presence of HCV-associated proteins within lymphoma cells has been documented [11]. It was found also that peripheral blood B-cells from patients with chronic HCV infection were infected and also had enhanced gene expression associated with B-cell NHL development when compared to healthy controls [12,13].

The accumulating epidemiological and molecular biological data supporting the theory for virally-induced lymphomagenesis in some individuals
with HCV infection are further reinforced by observation from series of clinical studies, involved in the biggest one 18 patients with primary splenic marginal zone lymphoma, 78% of the patients responded completely to antiviral treatment (Interferon and Ribavirin) and achieved sustained hematological and clinical response. Even patients with partial responses demonstrated varying degrees of hematological improvement proportional to the degree of reduction of viral load [14,15].

Bcl-2 gene product is an anti-apoptotic molecule that modulates the mitochondrial release of cytochrome c and the interaction of apoptosis activating factors with caspase 9 and Bax. Bcl-2 (a marker linked to germinal center B-cells) is located at chromosome 18q21. Bcl-2 overexpression provides a survival advantages for neoplastic B-cells and is thought to play an important role in resistance to chemotherapy and unfavorable prognosis [16].

The observation that treatment with interferon-α of hepatitis C virus positive patients with splenic marginal zone NHL results in HCV-clearance, reduced frequency of translocation t (14;18) and regression of NHL, clearly implies the causal role of HCV infection in at least certain subsets of NHL [14,17]. An interaction between genetics and environmental agents during lymphomagenesis has been suggested and HCV-infection could serve as an environmental stimulus in the development of lymphoma harboring the Bcl-2 rearrangement [17].

The present study was conducted to evaluate the expression of Bcl-2 gene in lymph node biopsy tissue samples from patients with NHL with and without HCV infection and to evaluate the association between Bcl-2 gene expression and the prognosis of NHL.

Patients and Methods

The study included 92 patients who attended the National Cancer Institute (NCI) Cairo University in the period between 2012-2014, 52 patients were diagnosed as B cell NHL with hepatitis C (23 female and 29 males) and a second group of 40 patients with B cell NHL without hepatitis C infection (19 females and 21 males). The age range was (30-70) years with mean age 49.2 for the first group and 43.6 for the second group. All NHL patients were diagnosed according to the REAL Classification and graded according to the WHO classification of lymphoma 2008 [18]. Lymph nodal (LN) tissues obtained from the patients were immediately cut into three parts; one piece was processed for routine histopathological examination to confirm the diagnosis and determine the tumor histological type, and grade, and for immunohistochemical study of Bcl-2. The second and third portions were snap-frozen and stored in liquid nitrogen for DNA and RNA extraction. Gene expression profiling was conducted with RNA samples from LN biopsies of the two groups. Gene expression profiling was performed using microarray chips (Fox Chase Cancer Center) containing 15500 human probes. Among them, the selected Bcl-2 gene either up-regulated or down-regulated was recorded based on greater than 2 folds ratio changes with significant p-value > 0.05. The RT-PCR was performed on RNA extracted from the patients according to manufacturer’s instructions to detect (HCV +ve) cases.

**cDNA Microarray:**

**RNA extraction from tissues:** RNA was prepared from tumors samples tissues, each sample was tested in triplicate on array 15k (Array 1) supplied form Fox Chase Cancer Center. RNA was extracted by homogenization (Polytronin; Kinematica, Luceme, Switzerland) in TRiZol reagent (Gibco BRL) at maximum speed for 90-120s. The homogenate was incubated for 5min at room temperature. A 1:5 volume of chloroform was added and the tube was vortexed and subjected to centrifugation at 12000g for 15min. The aqueous phase was isolated and one half of the volume of isopropanol was added to precipitate the RNA. Purification was then performed with the Qiagen RNeasy using total RNA isolation Kit according to manufacturer’s specification (Qiagen-Germany). The purified total RNA was finally eluted in 10ul of diethylpyrocarbonate treated H2O and the quantity and integrity were characterized using UV spectrophotometer (Nanodrop). RNA was electrophoresed on an ethidium bromide stained agarose gel. It showed discrete bands of high molecular weight RNA between 7kb & 15kb, two prominent ribosomal RNA bands at approximately 5kb & 2kb and low molecular weight RNA between 0.1 0.3kb. The isolated RNA has a 260/280 ratio of 1.9/2.1.

**RNA labeling:** Probes for microarray analysis were prepared from RNA template by the synthesis of first strand cDNA containing amino allyl-labeled nucleotides (Sigma Cat #A0410) followed by a covalent coupling labeled cDNA to the CyDye Ester to the NHS ester of the appropriate Cyanine flour Cy3-ester (Amersham Pharma Cat #PA23001) and Cy5-ester (Amersham Pharma Cat # A25001). This was followed by purification of the two probes by passing through Microtene 30 columns (Millipore-Bedford, MA) according to the manufacturer’s instructions.
**Hybridization:** Hybridization occurred in 1x hybridization buffer containing 50% form amide, 5x SSC and 0.1% SDS. Prior to hybridization, the free amino groups on the slide were blocked or inactivated in the pre-albumin (BSA, Sigma Cat # A94181, 5xSSC & 0.1%SDS).

**Data collection:** Primary data from image files were obtained using Scan Array Express II (Perkin Elmer, USA). A confocal Laser Scanner capable of interrogating both the Cy3- and Cy-5 labeled probes and producing separate images for each and then, normalized using intensity and spatially dependent method [19]. Following image processing, the data generated from the arrayed genes were further analyzed before differentially-expressed genes could be identified. We used Scan Array Express II (Perkin Elmer, USA) software for image processing. Measured intensities were analyzed using the Genesis software and R program that detects the up and down-regulated genes according to the ratio in their software’s [20]. The results were analyzed using GraphPad Prism Computer Program (GraphPad software, San Diego, USA). For genes expression analysis, Mann-Whitney test was used for numeric variables and Chi square or Fisher’s exact test was used to analyze categorical variables. The $p$-value was considered significant when $p<0.05$.

Antibodies to HCV were detected with HCV EIA version 3.0 (Innogenetics, Belgium). All serologic assays were done according to manufacturer instructions. Detection of HCV-RNA: RNA was extracted from patients’ sera and RT-PCR was performed according to manufacturer’s instructions by Qiagen (Qiagen, Gmb H, Germany).

**Immunohistochemical staining of BCL-2:** Five micron formalin-fixed paraffin- imbedded slides were prepared from tumor tissue from both groups of NHL with (52 patients) and without (40 patients) HCV infection. Anti-gene unmasking was performed by heat-induced epitoperetrieval method by placing the slides in citric acid buffer-filled jar in a microwave at 800 Watt for 20min (divided into 4 cycles, 5min each). The slides were rinsed three times in phosphate buffer saline (PBS). Endogenous peroxidase activity was blocked by incubation of the tissue sections with 3% hydrogen peroxide in water bath for 30min. After washing in PBS, the tissue sections were then incubated with the primary monoclonal AB, ready to use BCL-2 200 Ul for each slide for 2H at room temperature (clone 124, Dako, MO887) diluted at 1/40. Streptavidin biotin method was used as a detection kit (LSAB, Dako, Denmark). Tissue sections were incubated with biotinylated secondary AB for 40min then with Streptavidin conjugated enzyme for 30min during which the 3, 3 diaminobenzidine (DAB) substrate chromogen was freshly prepared then added onto the tissue sections for 10 Min. The tissue sections were counterstained in Meyer’s Haematoxylin. Immunohistochemical staining results were scored semi-quantitatively. The positivity cut off was considered when the reactivity of lymphoma cells with BCL-2 stain was more than 20%. The membranous and cytoplasmic patterns were considered positive. The staining results were analyzed using SPSS program, version 13, chi square test with $p$-value $\leq 0.05$ [21]. The study protocol was conformed to the ethical guidelines and was approved by Sohag University Medical ethical committee.

**Results**

This hospital-based, case-control study was done at Department of pathology, Sohag University Hospital, and National Cancer Institute, Cairo University. Ninety two patients were involved in the study divided into 52 cases of B-cell NHL with HCV +ve and 40 cases of B-cell NHL with HCV –ve. The age range was 30-70 years for both groups with mean age 49.5 for HCV +ve and 43.6 for HCV –ve cases as shown in Table (1). Three type of B-cell NHL were included in the study, DLBCL formed 54.3% (50 cases) of the studied cases followed by FL, 26.1% (24 cases) and SLL, 19.6% (18 cases) as showed in Fig. (1). HCV +ve cases constituted 56.5% of the study cases. There was no statistically significant correlation between HCV infection and specific NHL type as DLBCL with HCV appeared in 53.8% of the cases while DLBCL HCV –ve formed 55% of the cases, FL with HCV appeared in 26.9% and HCV –ve FL formed 25% of the cases, SLL with HCV +ve formed 19.2% while SLL with HCV-ve constituted 20% of the cases as shown in Fig. (1).

Bcl-2 was expressed in 25 cases of the studied patients forming 27% of the cases. Bcl-2 was expressed in NHL with HCV +ve patients 32.7% more than NHL with HCV –ve cases 20% as shown in Fig. (2) with statistically significant difference $p$-value 0.003. There was also a statistically significant correlation between Bcl-2 expression and grade of the NHL as Bcl-2 was expressed in High grade NHL (DLBCL) 19.2% more than Low grade (FL and SLL) 13.5% with $p$-value 0.003. There was no significant correlation between Bcl-2 expression and specific type of NHL. There was no correlation between different prognostic indicators and the HCV infection or the Bcl-2 expression Table (1), although the number of the cases in the study was too small to draw conclusions.
Table (1): The clinicopathological features of the studied groups.

<table>
<thead>
<tr>
<th></th>
<th>NHL HCV +ve</th>
<th>NHL HCV –ve</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Age range</td>
<td>30-70</td>
<td>30-70</td>
<td></td>
</tr>
<tr>
<td>Mean age</td>
<td>49.5</td>
<td>43.6</td>
<td></td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>♀</td>
<td>23</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>♂</td>
<td>29</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52 (56.5%)</td>
<td>40 (43.4%)</td>
<td></td>
</tr>
<tr>
<td>IPI score:</td>
<td></td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>Age &lt;60</td>
<td>18</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Stage III &amp; IV</td>
<td>29 (55.8%)</td>
<td>26 (65%)</td>
<td></td>
</tr>
<tr>
<td>Performance &lt;2</td>
<td>28 (53.8%)</td>
<td>20 (50%)</td>
<td></td>
</tr>
<tr>
<td>DFS (24m)</td>
<td>66%</td>
<td>80%</td>
<td></td>
</tr>
</tbody>
</table>

IPI: International prognostic indicators. DFS: Disease free survival.

Table (2): Bcl-2 expression results in the studied patients.

<table>
<thead>
<tr>
<th></th>
<th>NHL HCV +ve</th>
<th>NHL HCV –ve</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLBCL</td>
<td>28</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>14</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>SLL</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Bcl-2+ve</td>
<td></td>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>DLBCL</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>SLL</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 (32.7%)</td>
<td>8 (20%)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

DLBCL: Diffuse large B cell lymphoma.
FL: Follicular lymphoma.
SLL: Small lymphocytic lymphoma.
HCV: Hepatitis C virus.

Figure

(A): CD20 +ve immunostaining in FL.
(B): Bcl-2 expression in FL.
(C): Strong Bcl-2 expression in FL.
(D): DLBCL stained by (H&E).
(E): DLBCL showed CD20 +ve immunostaining.
(F): Strong Bcl-2 +ve staining in DLBCL.
Discussion

The mechanism of lymphomagenesis in hepatitis C virus related NHL B-cell type is unknown. The research efforts focused on hepatitis C virus-induced NHL are essential to properly understand the virus induced lymphomagenic mechanisms in order to develop effective intervention strategies and to reduce the disease burden.

Hepatitis C infected NHL patients constituted 56.6% of the studied cases while HCV –ve NHL cases formed 43.4% of the cases.

Available studies apparently supported the possibility of oncogenic role of HCV in the development of NHL [22,23].

It has been demonstrated that acute and chronic HCV infection causes a 5-10 fold increase in mutation frequency in Ig heavy chain, Bcl-6, P53 and $\beta$-catenin genes of in vitro HCV-infected B-cell lines and lymphomas [24].

It was reported that engagement of CD81 on human B-cells by a combination of HCV-E2 protein leads to the proliferation of Naïve B-cells and E2-CD81 interaction induces protein tyrosine phosphorylation and hypermutation of immunoglobulin genes in B-cell lines. The engagement of CD81 resulted in polyclonal expression of naïve CD27-ve B-cells. Consequently, chronic antigenic stimulation may play a significant role in the development of an initial polyclonal B-cell expansion which may progress to autonomous B-cell proliferation, immune dysregulation and eventually B-cell malignancy [25].

Analysis of 7 studies from International lymphoma Epidemiology Consortium (Inter Lymph) based in Europe, North America and Australia found that HCV infection was detected in (3.6%) (172/4784 cases) NHL cases and in (2.7%) (169/6269 cases) of the control group. They concluded a similar result to our study that there was an association between HCV infection and NHL special types; diffuse large B-cell lymphoma, marginal zone lymphoma and lymphoplasmacytic lymphoma. These increasing evidences indicated that the association between HCV infection and lymphoma may be owing to viral infection. The chronic inflammation pathway would be consistent with regression of some lymphoma after eradicating the HCV infection [26]. A recent study found that HCV infection is (2-3 times) more prevalent in NHL patients (292 cases) than the control group (1168 cases) [27].

The present study demonstrated a significant up-regulation in Bcl-2 both by tissue microarray and immunohistochemically in (32%) of patients with NHL with hepatitis C compared to (20%) in NHL patients without hepatitis C infection.

Different studies have showed a significant association between HCV infection and Bcl-2 rearrangement t(14; 18) translocation. In these patients, clonal expression of B-cell harboring t(14; 18) was demonstrated with overexpression of the anti-apoptotic Bcl-2 protein resulting in higher Bcl-2/Bax ratio [28-32].

Although the number of patients investigated in this study was too small to draw definitive conclusions, there was a correlation between HCV infection NHL and the poor prognostic indicators and the patient performance, however, there was no statistically significant correlation between them.

In agreement with our results, Sohan et al found that Bcl-2 expression was identified in 24/92 (26.4%) patients of DLBCL and Bcl-2 expression was found to be unfavorably associated with the OS in a confined group with low or low intermediate IPI scores and such patients could be candidates for more intensive therapy or alternative therapeutic approaches [33].

Iqbal et al., found that Bcl-2 rearrangement with the t(14; 18) (q32; q21) was detected in approximately (20%) of DLBCL patients and concluded that Bcl-2 plays a major role in the response of malignant cells to a variety of stresses that may lead to apoptosis including chemotherapy while El-Esawy found that Bcl-2 is expressed in (42.4%) of cases of B-cell NHL especially low grade follicular lymphoma and is associated with poor prognostic indicators (old age, low platelets count, high serum LDH (Lactic dehydrogenase enzyme) and elevated total leukocytes count (TLC) [34,35].

Bcl-2/IgH was detected in peripheral mononuclear cells by nested PCR in (38.2%) of chronically infected HCV patients [36]. These results with ours supported the idea that HCV may play a role in malignant transformation and lymphoma genesis in patients with chronic hepatitis C infection.

On contradictory to our findings, other studies found that HCV infected NHL patients did not have a higher frequency of Bcl-2 rearrangement than HCV-negative NHL patients and t(14; 18) translocation in PBMCs (Peripheral Blood mononuclear cells) from HCV infected patients does not appear to be a predictive marker of lymphomagenesis [37-39].
The t(14;18) in B lymphocytes is not itself a transforming factor but it is considered a step in the lymphomagenesis of some types of NHL as follicular lymphoma and DLBCL. It may be affected by sustained viral antigenic stimulation as in patients with hepatitis C +ve NHL. [29-38].

It is understandable that different mechanisms proposed as chronic antigenic stimulation, interaction between HCV-E2 protein and its cellular receptor (CD81) on B-cell and direct HCV infection of B-cells may be combined together in multifactorial model of HCV-associated lymphomagenesis [8]. Whatever the mechanism of deregulation of the Bcl-2 gene, the finding of a high expression of Bcl-2 observed in a large proportion of NHL raises the question of its clinical value and biological significance, because Bcl-2 is now regarded as a member of a new category of oncogenes involved in blocking programmed cell death, thus leading to abnormal accumulation of cells.

Conclusions:

It is understandable that different mechanisms proposed in the process of lymphomagenesis, one of them is Bcl-2 gene mutation especially in NHL with HCV infected patients. It is useful to use this finding in modulating the treatment of those patients using antiviral therapy in addition to chemotherapy regimen.

References


ملخص البحث:

الأندية[idx] حديثاً سرطان الغدد الليمفاوية في مرضى الالتهاب الكبدى الفيروسي في غير معروفة من المقترح أن الفيروس الكبدى س قد يسبب تكاثر في عدد الخلايا الليمفاوية وظهور العامل ب س ل في المرضى المصابين بالالتهاب الكبدى الفيروسي المزمن.

الهدف من البحث:

تقييم ظهور العامل ب س ل في مرضى سرطان الغدد الليمفاوية المصابين بالالتهاب الكبدى الفيروسي.

طريقة البحث:

تستند الدراسة 92 مريض منهم 52 مريض بسرطان الغدد الليمفاوية بالالتهاب الكبدى الفيروسي المزمن و 40 مريض بسرطان الغدد الليمفاوية بدون الالتهاب الكبدى الفيروسي المزمن على المعهد القومي للأورام بالقاهرة خلال الفترة من مارس 2012 إلى مايو 2014. تم فحص عينات من الخلايا الليمفاوية لجميع المرضى لتقديم ظهور العامل ب س ل باستخدام طريقة الفحص المناعي الكيميائي والفحص النسيجي بجهاز اليمكواري.

نتائج البحث:

تثبت الدراسة أن 200.7% من حالات سرطان الغدد الليمفاوية باءت بالالتهاب الكبدى الفيروسي المزمن كيف.7% فقط من حالات سرطان الغدد الليمفاوية بدون الالتهاب الكبدى الفيروسي المزمن في استخدام طريقة الفحص المناعي الكيميائي النسيجي الكيميائي.

كما وجد ان العامل ب س ل تضايق باكثر من مرضى في مرضى سرطان الغدد الليمفاوية والالتهاب الكبدى الفيروسي المزمن.

الاستنتاج:

تظهر صورة أكبر في مرضى سرطان الغدد الليمفاوية والمصابين بالالتهاب الكبدى الفيروسي المزمن عنا في مرضى سرطان الغدد الليمفاوية وغير مصابين بالالتهاب الكبدى الفيروسي المزمن وقد يلعب دورا في عملية تكوين سرطان العقد الليمفاوية وكذلك في سبيل علاج في المستقبل.