**Frequency and Clinical Association of NY-ESO-1 Gene Expression in Diffuse Large B-cell Lymphoma.**

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- Translational Medicine

**KEYWORDS**
- DLBCL = Diffuse Large B-cell Lymphoma, NY-ESO-1, qRT-PCR = quantitative real-time polymerase chain reaction
Abstract

Background

Diffuse Large B-cell Lymphoma, a heterogeneous disease, is influenced by complex network of gene interactions. In current study, we aimed to evaluate the frequency of expression and determine the expression levels of the NY-ESO-1 gene in patients with DLBCL, as well as to examine its relationship with clinical parameters and survival.

Methods

We analyzed NY-ESO-1 gene expression levels using real-time quantitative RT-PCR (qRT-PCR) in 112 patients with DLBCL. The associations between the expression of the NY-ESO-1 gene and the clinical variables were evaluated using the chi-square test and Fisher’s exact test. Overall survival (OS) was determined using the Kaplan-Meier method.

Result

The results showed that the NY-ESO-1 gene was expressed in 46.4% (52/112) of patients with DLBCL, and NY-ESO-1 gene expression was associated with clinical parameters such as LDH, clinical stage, international prognostic index (IPI) (p≤0.05). High levels of NY-ESO-1 gene expression were correlated with advanced disease stages, and the survival rates after 5.3 years of tracking were lower in the patients expressing the NY-ESO-1 gene (66.4%) than in those not expressing the gene (23.1%).

Conclusion

In conclusion, the expression levels of the NY-ESO-1 gene in patients with DLBCL may be of great utility for diagnosing and determining the prognosis of this disease.

Background

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma, and it is responsible for approximately 30 to 50% of all new cases.\(^1\,^2\) DLBCL represents a heterogeneous group of tumors with highly variable genetic abnormalities, clinical characteristics, responses to treatment, and prognosis.\(^3\,^4\,^5\) The diagnosis of DLBCL is accomplished through histopathological studies and immunophenotyping. The following clinical criteria are currently used for determining the prognosis of this disease: clinical stage, functional state (ECOG), international prognostic index (IPI), LDH levels,
and β2 microglobulin levels. Despite advances in immunotherapy (anti-CD20 therapy), as well as the incorporation of new cytotoxic agents (bendamustine), a select group of patients continue to have an unfavorable prognoses.6

The subdivision of DLBCL into two major biological categories based on their presumed cell of origin: germinal center B-cell (GCB), and activated B-cell (ABC).4-5

Several molecular alterations have been identified in DLBCL, such as the expression abnormal of the NY-ESO-1 gene (New York esophageal squamous cell carcinoma-1), which is part of the group of cancer-testis antigen (CTA). This gene encodes a protein that is overexpressed in many cancers, but absent in normal tissue except for testicular. This gene is found in a duplicated region of the X-chromosome and therefore has a neighboring gene of identical sequence. It has been used to diagnose and assess the prognosis of various types of cancer,8-10 and its expression is restricted solely to immune-privileged germinal cells, which are the most immunogenic of this family.11,12 It is abnormally expressed in a variety of cancers and is associated with the unfavorable evolution of multiple myeloma16,17 and non-small cell lung cancer.13-14

Our group analyzed the NY-ESO-1 gene expression levels of patients with DLBCL using quantitative in real-time (qRT-PCR) and demonstrated that there is a relationship between the clinical parameters and a lower survival rate, the detection of NY-ESO-1 by qRT-PCR could be useful for disease prognosis and follow-up

Methods

Study design

Prospective, observational and experimental clinical study. This was a prospective clinical study with 112 patients with DLBCL who had previously provided signed informed consent forms. The histological diagnosis was established according to the World Health Organization (WHO) classification (SH, 2008). The study population was characterized according to their clinical parameters, including prior medical history, disease stage, and levels of lactate dehydrogenases (LDH). The average age was 45 years (range 18 to 69), and 46.4% were male and 53.5% female. The patients were treated with R-CHOP
(Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone). The patients showing a partial response were treated with dexamethasone, etoposide, and cisplatin as second-line chemotherapy at the discretion of the treating doctor. The survival global analysis was conducted after 5.3 years. This study clinical was approved by the Ethics, Research and Biosafety Committees of the General Hospital of Mexico "Dr. Eduardo Liceaga" DI/19/103/03/006. The study adhered to the principles of the recent revision to the Declaration of Helsinki. All participants provided signed forms indicating prior consent. Lymph nodes from the patients were frozen in liquid nitrogen immediately after surgical excision and stored until RNA extraction. Testicular tissue from a 60-year-old patient with prostate cancer was used, to determine the levels of relative expression basal of the NY-ESO-1 gene.

**Analysis of NY-ESO expression**

Expression levels of the NY-ESO-1 gene were evaluated prior to treatment initiation in both treatment groups. Total cellular RNA was extracted from the frozen tissue and the controls using TRIzol® Reagent (Thermo Fisher Scientific Inc). The RNA was stored at -80°C until needed. A total of 2µg of RNA was used for the synthesis of cDNA by means of the reverse transcriptase M-MLV

**Real-time polymerase chain reaction (qRT-PCR) analysis.**

The mRNA expression levels of the NY-ESO-1 (Hs00265824_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs00985689) genes were measured using the TaqMan® gene expression assay (Applied Biosystems, Foster City, CA, USA). The GAPDH gene was used as an endogenous control, and each sample was analyzed in triplicate. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method using Testicular tissue as calibrator. We used the median as cut-off between high and low expression.

**Statistical analysis**

The correlation analyses between NY-ESO-1 gene expression and the clinical variables were performed using the chi-square test and Fisher’s exact test. The survival data were analyzed using the Kaplan-Meier method and compared with the log-rank test, considering a p-value ≤ 0.05 to be
The statistical program S.P.S.S. version 20 (Statistical Package for Social Sciences, SPSS Inc., Chicago, USA) was used for the analyses.

**Results**

**Frequency of NY-ESO-1 expression at the mRNA level in DLBCL patients**

The frequency of NY-ESO-1 gene expression was 46.4% (52/112). The levels of relative expression with respect to control (testicular tissue) were 0.2 times in stages I/II, while in stages III / IV they were 1.5 times and 2.2 times, respectively Figure 1. The expression levels were significantly different between stages III y IV in comparison with stage I/II, revealing a relationship between the level of expression and advanced-stage disease (p= 0.007).

**Association of NY-ESO-1 expression with prognostic parameters**

The statistical analysis showed significant values for the parameters of LDH, clinical stage, and IPI (p≤0.05). Elevated LDH levels in serum and a high IPI were associated with gene expression in 39.2% (p=0.001) and 32.1% (p= 0.019) of the patients, respectively. Additionally, 42.8% of the positives were associated with clinical stage III or IV (p=0.001) Table 1.

**Expression of NY-ESO-1 and its relation to the survival rate**

The study was performed over 5.3 years, and survival median at 3 years was 23.1% for the positive patients and 66.4% for the negative patients (Figure 2). During this period, we observed that 76.9% (40/52) of the patients expressing NY-ESO-1 died. In contrast, 33.3% (20/60) of the negative patients died. In the statistical analysis, a log-rank value of p= 0.001 was calculated.

**Discussion**

Patients with DLBCL exhibit heterogeneous clinical characteristics, as well as variability in their responses to treatment and prognoses.15-16 Although survival can be estimated based on clinical parameters (age, LDH levels in serum, extranodal site involvement, disease stage, and immunophenotype B), as well as molecular abnormalities (p53, BCL-2, BCL6, MUM.1 and Ki67), controversy exists regarding their utility as prognostic and survival markers.17 As a result, it is of paramount importance to find new markers that could be incorporated to determine the prognosis of this disease.
We evaluated the clinic pathological relevance of NY-ESO-1 gene expression in patients with DLBCL at diagnosis who were admitted to the Hematology service of the Hospital General de México. We decided to examine the expression of the NY-ESO-1 gene in patients with lymphoma, as it is a CTA present in various types of cancer and is associated with clinical factors such as poor prognosis and lower survival.\textsuperscript{18–19} We confirmed that NY-ESO-1 gene expression is associated with the advanced stage of the disease, changes in the levels of LDH and the IPI, and survival rates. In DLBCL, there are no reports of an association between the expression of this gene and clinical parameters. Hudolin et al.\textsuperscript{20} analyzed the expression of the NY-ESO-1 gene in 24 samples of testicular tissue with DLBCL; expression was observed in 54.1% and was not correlated with clinical parameters or survival. Other reported molecular markers for DLBCL include p53, bcl-2, and ki67, but these markers were not associated with clinical parameters.\textsuperscript{17}

We observed increases in the frequency of expression and the amount of NY-ESO-1 gene transcription with disease stage in patients with DLBCL, with increases of 3.5% in stages I-II and up to 42.8% in stages III-IV. These results are consistent with previous reports on melanoma in which a 3.34% increase in this gene in stage I and a 9.52% increase in stage II were observed, as was an increase of up to 45% in stage III.\textsuperscript{21} Similar results were reported in bladder and prostate cancer, where the frequency of expression increases with respect to the stage of the disease.\textsuperscript{22–23} Only 2 studies have measured the expression levels in metastatic esophageal squamous cell carcinoma and non-small cell lung cancer using qRT-PCR, and elevated transcription levels were associated with advanced disease stages.\textsuperscript{24–25} In the past, our group demonstrated an association between transcription of the MAGE-A3 gene and advanced stages in patients with DLBCL and leukemia.\textsuperscript{26–27}

In some patients, the MAGE-A3 gene was co-expressed with the NY-ESO-1 gene, which may indicate an unfavorable prognosis. The increase in the level of NY-ESO-1 gene transcription in patients with DLBCL is a finding of great importance; It could be a prognostic marker for this disease. Additionally, the increase in advanced stages of the disease may explain its oncogenic role and the proliferative advantage it confers to tumor cells.
Global survival is lower in patients who express NY-ESO-1, and these results concur with those reported for lung cancer, demonstrating that the expression of NY-ESO-1 is significantly associated with an adverse prognosis. Similar data associating the expression of this gene with decreased disease-free survival have been reported for gastrointestinal and bladder cancer. Other reports have examined the associations between the expression of the p53, bcl-2, and ki67 genes and global survival in patients with DLBCL and did not observe an association. Rearrangements of the BCL-6 gene have been associated with 50% survival at 5 years in patients treated with R-CHOP, although the reported expression frequency was only 19%. We have reported that the MAGE-A3 gene is associated with a decrease in survival in patients with DLBCL. Thus, the expression of the MAGE-A3 and NY-ESO-1 genes may have great utility for predicting survival in patients with DLBCL.

Similar reports have examined the expression of the MAGE-A2 gene, which inhibits the function of p53 through the recruitment of histone deacetylases and confers resistance to etoposide. Another study has demonstrated that in patients with DLBCL who were treated with anthracyclines, chemoresistance was associated with high expression levels of the PRAME gene, which is another member of the CTA family, as well as with a lower global survival.

NY-ESO-1 gene expression in patients with DLBCL may be helpful for identifying and stratifying risk groups, with other molecular marker of this disease that may benefit from new or intensified therapies.

**Conclusion**

In conclusion, our results demonstrate that expression of the NY-ESO-1 gene is associated with a poor prognosis of patients with DLBCL, and it is highly important to incorporate this gene into panels of existing molecular markers.

**Abbreviations**

- **DLBCL**: Diffuse Large B-cell Lymphoma,
- **qRT-PCR**: real-time quantitative polymerase chain reaction
- **WHO**: World Health Organization
**dNTP:** deoxy-ribonucleotide triphosphate

**IPI:** international prognostic index

**GCB:** germinal center B-cell

**GADPH:** glyceraldehyde 3-phosphate dehydrogenase

**CR:** complete remission

**RFS:** relapse-free survival

**OS:** overall survival

**FAB:** French-Britain-American

**WBC:** white blood cell

**OR:** odds ratio

**HR:** hazard ratio

**CI:** confidence interval

Declarations

**Authors’ contributions**

IOC made main contributions to the document, designed the research, and participated in specimen collection, sorting data, data analysis and drafting the manuscript; CRP, EMP, EGG and JCJ assisted in specimen collection and the acquisition of clinical data; AMT provided substantial guidance and revised the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of date and materials**
The datasets supporting the conclusions of this article are included within the article (and its additional files).

Consent for publication

Not applicable.

Ethics approval and consent to participate This study was approved by the Ethics Committee of the General Hospital of Mexico (Registration Number: HGM/DI/19/103/03/006)

Clinical and informed consent was obtained from each patient or their family members.

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Table 1. Associations between clinical characteristics and NY-ESO-1 expression in DLBCL patients.

|                      | Positive | Negative |
|----------------------|----------|----------|
| **Age**              |          |          |
| Median (Range)       | 58(18-69)| 37(19-65) |
| **Sex**              |          |          |
| Male                 | 20(17.8) | 32(28.5) |
| Female               | 32(28.5) | 28(25)   |
| **More than 1 extranodal site** | | |
| Yes                  | 32(28.5) | 20(17.85) |
| No                   | 20(17.8) | 40(35.7) |
| **ECOG performance status greater** | | |
| 0                    | 0        | 4(3.5)   |
| 1                    | 28(25)   | 48(42.8) |
| 2                    | 24(21.4) | 8(7)     |
| **Serum LDH level > normal** | | |
| Yes                  | 44(39.2) | 12(10.7) |
| No                   | 8(7)     | 48(42.8) |
| **IPI**              |          |          |
| Low                  | 4(3.5)   | 28(25)   |
| Low-intermediate     | 12(10.7) | 20(17.8) |
| High                 | 36(32.1) | 12(10.7) |
| **Stage**            |          |          |
| I-II                 | 4(3.5)   | 40(35.7) |
| III-IV               | 48(42.8) | 20(17.8) |

*Statistically significant at the 0.05 level.
* Significance level $p \leq 0.05$

LDH: Lactate dehydrogenase ( % )

Figures
mRNA expression levels of NY-ESO 1 in DLCBL stages. The expression results were obtained from 4 samples from stage I and II, 48 from stage III and IV. Significant differences between results (p ≤ 0.5) were obtained by means of parametric test. T, testicular tissue.
Figure 2

Overall survival in DLCBL patients based on the NY-ESO-1 expression. The 5.3 years overall survival rate is analyzed in patients. Survival median at 3 years was 23.1 for the positive and 66.4% for the negative patients (p=0.001).