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ORIGINAL ARTICLE

Cancer/testis antigens expression and autologous serological response in a set of Brazilian non-Hodgkin's lymphoma patients

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Abstract
Background Based on their tumor-associated expression pattern, cancer/testis antigens (CTAs) are considered potential targets for cancer immunotherapy. We aim to evaluate the expression of CTAs in non-Hodgkin's lymphoma (NHL) samples and the ability of these patients to elicit spontaneous humoral immune response against CTAs.
Methods Expression of MAGE-A family, CT7/MAGE-C1, CT10/MAGE-C2, GAGE and NY-ESO-1 was analyzed by immunohistochemistry in a tissue microarray generated from 106 NHL archival cases. The humoral response against 19 CTAs was tested in 97 untreated NHL serum samples using ELISA technique.
Results 11.3 % of NHL tumor samples expressed at least 1 CTA. MAGE-A family (6.6 %), GAGE (5.7 %) and NY-ESO-1(4.7 %) were the most frequently expressed antigens. We found no statistically significant correlation between CTA positivity and clinical parameters such as NHL histological subtype, Ann Arbor stage, international prognostic index score, response to treatment and overall survival. Humoral response against at least 1 CTA was observed in 16.5 % of NHL serum samples. However, overall seroreactivity was low, and strong titers (>1:1000) were observed in only two diffuse large B-cell lymphomas patients against CT45.
Conclusion Our findings are in agreement with most of published studies in this field to date and suggest an overall low expression of CTAs in NHL patients. However, as many new CTAs have been described recently and some of them are found to be highly expressed in NHL cell lines and tumor samples, further studies exploring the expression of different panels of CTAs are needed to evaluate their role as candidates for immunotherapy in NHL patients.

Keywords Non-Hodgkin's lymphoma · Cancer/testis antigens · Humoral response

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Background

The search for tumor-specific antigens as potential targets for immunotherapeutic approach has been challenging for several decades. Cancer/testis antigens (CTAs) have emerged as attractive candidates for cancer-specific immunotherapy due to their particular characteristics of high immunogenicity with no or highly restricted expression in normal tissues [1]. Several studies have demonstrated that these antigens are able to elicit specific humoral

and T-cell-mediated cytotoxic immune responses in cancer patients, pointing them as possible cancer vaccine targets. One common feature of CTA expression is its induction by the DNA methyl-transferase 1 inhibitors, 5-aza-2-deoxycytidine and/or by histone deacetylase inhibitors [16–18]. These findings, together with the inclination for global hypomethylation in cancer, suggest that CpG island hypomethylation at the promoter regions of these genes is the possible mechanism for transcriptional activation of CTA genes in cancer [1]. There are more than 100 CTA genes reported in the literature to date [2]. About 30 of these CTA genes are encoded by multigene families on chromosome X (classical CTAs) [3]. These CTAs are of particular interest because almost all CT-X present higher immunogenicity and highly restricted expression pattern in normal tissues compared to those not encoded on chromosome X (non-classical CTAs). Some non-classical CTAs like SCP-1, [4], OY-TES-1 [5], SPO11 [6] and BORIS [7] have an established role in gametogenesis, but in the tumorigenesis field, the biological function of most CTAs remains poorly understood. Recent studies provided some evidence that CTAs may have antiapoptotic properties rather than regulating cell proliferation or adhesion in cancer. [8–15]. The frequency of CTA expression is highly variable among different tumor types: melanoma, ovarian cancer and lung cancer are considered tumors with high frequency of CTA expression, while hematopoietic malignancies, renal, colon and pancreatic cancers, have been described as tumors with low frequency of CTA expression [1]. Some exceptions to this observation among hematopoietic malignancies are the high expression of CT7/MAGE-C1 in multiple myeloma [19, 20], and CT45 in classical Hodgkin lymphoma [21, 22]. Studies correlating CTA expression with clinicopathological features in different tumor types have demonstrated the association of CTA positivity with higher tumor grade, advanced stage or metastatic disease and worse clinical outcome [19, 23–31]. Some studies performed in cancer cell lines have suggested the correlation of CTA expression with resistance to some antineoplastic agents and gamma-irradiation. It could explain, in part, the poor prognosis of these patients [15, 32].

Despite being frequent hematologic malignancy, there are few studies evaluating the expression of CTAs in non-Hodgkin's lymphoma (NHL) to date. The largest study accessing CTA expression in NHL analyzed the expression of 8 classical and non-classical CTAs (*MAGE-A3, MAGE-A4, CT7, SSX-1, SSX-2, SSX-4, SCP-1* and *HOM-TES-85*) using RT-PCR technique in 93 NHL samples [33]. It was demonstrated that diffuse large B-cell lymphomas (DLBCL), a subtype of B-cell lymphoma, showed the highest frequency of CTA expression. *SCP-1* (7/28), *SSX-1* (5/28) and *CT7* (2/28) were the CTAs more frequently expressed in this subgroup, and among T-cell lymphomas, the majority of samples (9/15) expressed *SCP-1* (6/9 peripheral T-cell lymphomas, 2/4 angioimmunoblastic lymphoma and 1/2 precursor T-cell lymphoblastic lymphoma). Another study demonstrated a significant cytotoxic T-cell response in 21/29 HLA-A*0201-positive DLBCL patients with CT63/PASD1 expression using SEREX (Serological Analysis of Recombinant cDNA Expression) technique, identifying CT63/PASD1 as one of the most important candidates for cancer vaccines in DLBCL [34–36]. Recently, Chen et al. [21] also described the expression of CT45 in 42/72 (58 %) of classical Hodgkin's lymphoma (cHL) and 28/126 (22 %) of DLBCL samples by immunohistochemistry. Interestingly, despite the remarkable high CT45 expression in cHL, only 1 of 67 patients had detectable anti-CT45 antibodies, suggesting low immunogenicity of this CTA or a suppressed immune response in cHL patients. Except for the CTAs described above, the available studies suggest an overall low expression of CTA in NHL.

Considering that the available information about CTA expression in lymphomas is scarce and heterogeneous regarding methods and samples, we immunohistochemically investigated the protein expression of a panel of CTAs in NHL tissue samples. We also studied the spontaneous humoral immune response in sera of NHL patients to evaluate the potential of CTAs as prognostic markers and candidates for immunotherapeutic approach in NHL patients.

Patients and methods

We retrospectively reviewed all cases of NHL diagnosed between 2003 and 2007 at the Hematology Service of Universidade Federal de Sao Paulo. The histology was reviewed by an experienced hemopathologist (A.C.A.), and NHL cases with sufficient material in paraffin blocks for tissue microarray (TMA) construction were included in this study. All patients included in this study were staged, classified according to the international prognostic index (IPI) and treated according to NHL treatment guidelines available from 2003 to 2007 [37]. Due to unavailability of rituximab in our public hospital at this time, B-cell lymphoma patients were uniformly treated with CHOP-like chemotherapy regimens without anti-CD20 monoclonal antibody.

This research was submitted to the Brazilian Research Council and approved by the Ethical Review Committee of our Institution according to the Declaration of Helsinki (Ethics Committee Approval 0998/07), and all patients provided written informed consent.

Tissue microarray (TMA)

Formalin-fixed paraffin-embedded tissues of 106 previously untreated NHL patients were obtained from the archives of the Department of Pathology, Hospital São Paulo, UNIFESP, Brazil. According to the World Health Organization (WHO) classification [38], the NHL cases consisted of 56 DLBCL, 10 follicular lymphomas, 9 peripheral T-cell lymphomas, 7 small lymphocytic lymphomas, 5 MALT (mucosa-associated lymphoid tissues) lymphomas, 4 marginal zone lymphomas, 3 lymphoplasmacytic lymphomas, 3 T-cell lymphoblastic lymphomas, 2 B-cell lymphoblastic lymphomas, 2 mantle cell lymphomas, 2 anaplastic large cell lymphomas, 2 mycosis fungoides and 1 adult T-cell leukemia/lymphoma. Table 1 summarizes the clinical data of these patients. Slides from all cases were reviewed and representative blocks were chosen for tissue microarray assembly.

Core-needle biopsies of paraffin-embedded tissue were obtained and then re-embedded in an array master block using techniques originally developed by Kononen et al. [39] and then modified by Hedvat et al. [40]. A Beecher Instruments (Sun Prairie, WI, USA) arraying device was used to assemble the arrays. Three core-needle biopsies (1.0 mm diameter) from tumor representative areas of each NHL case were included on tissue microarray blocks.

All CTAs chosen for immunohistochemical analysis in this study were classical CTAs due to their higher immunogenicity and highly restricted expression in normal tissues. The following monoclonal antibodies (to the following antigens) were included in our panel: MA454 (MAGE-A1), M3H67 (several MAGE-A antigens), 57B (MAGE-A1, -A3, -A4, -A6 and -A12), MAGE-A10#9 (MAGE-A10), CT7-33 (CT7/MAGE-C1), CT10#5 (CT10/MAGE-C2), clone #26 (GAGE-family, B&D Transduction Labs, Lexington, KY) and E978 (NY-ESO-1). The specificity of each antibody has been tested in previous studies [28, 41–48]. A heat-based antigen-retrieval method was used for all antibodies (vegetable steamer [Oster-Sunbeam, Ft. Lauderdale, FL, USA] 90 °C, 30 min). Except for mAb E978, a biotinylated horse anti-mouse secondary antibody (1:200; Vector, Burlingame, CA, USA) was used to detect primary antibody, followed by an avidin–biotin system (ABC-elite kit, Vector). E978 was detected with Immunovision kit (Leica Microsystems, Buffalo Grove, MN, USA). 3,3′-Diaminobenzidine tetrahydrochloride (Biogenex; San Ramon, CA, USA) served as chromogen. Endogenous peroxidase was suppressed by 1 % H_2O_2 for 20 min. The extent of tumor staining was graded based on the amount of immunopositive tumor cells as follows: ≤25 %: +, 26–50 %: ++, 51–75 %: +++ and 76–100 %: ++++). Testis with preserved spermatogenesis was used as positive controls, and reactive lymph nodes and tonsils samples were used as negative controls for all antibodies.

Table 1 Clinical data of non-Hodgkin's lymphoma patients included in TMA study ($n = 106$)

Clinical data	*n*	%
Age		
≤60	67	63.2
>60	39	36.8
Median age: 54 (17–91)		
Gender		
Male	55	51.9
Female	51	48.1
HIV status		
Negative	97	91.5
Positive	9	8.5
Tumor cell origin		
B-cell	89	84.0
T-cell	16	15.1
Non-B/non-T	1	0.9
Clinical behavior		
Indolent	32	30.2
Aggressive	74	69.8
DLBCL/Non-DLBCL		
Non-DLBCL	50	47.2
DLBCL	56	52.8
Ann Arbor staging		
Early (I or II)	41	38.7
Advanced (III or IV)	65	61.3
IPI		
Low	31	29.2
Intermediate (low and high intermediate)	46	43.4
High	19	17.9
Not available	10	9.4
Response		
Complete response	44	41.5
Partial response	22	20.8
Stable disease	0	0
Progressive disease	38	35.8
Not available	2	1.9

IPI international prognostic index

Spontaneous humoral immune response analysis by ELISA

Serum samples of 97 NHL cases, including 59 cases from the TMA cohort, were from patients referred from Hospital Sao Paulo, UNIFESP, Brazil. All samples were obtained at diagnosis, before any therapeutic interventions, to avoid treatment-related influences on humoral immune response analyses. According to the WHO classification, the NHL

cases consisted of 50 DLBCL, 10 follicular lymphomas, 8 peripheral T-cell lymphomas, 6 MALT lymphomas, 5 marginal zone lymphomas, 4 mantle cell lymphomas, 4 adult T-cell leukemia/lymphoma, 3 small lymphocytic lymphomas, 2 T-cell lymphoblastic lymphomas, 2 B-cell lymphoblastic lymphomas, 2 anaplastic large cell lymphomas and 1 mycosis fungoides. Clinical data of NHL patients included in spontaneous humoral immune response analysis are summarized in Table 2.

Serum samples were screened for the presence of IgG-specific responses against MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, NY-ESO-1, CT7, CT10, CT24, CT45, CT46, CT63, CT83, SSX-1, SSX-2, SSX-4, LAGE-1, GAGE-2, SAGE-1 and XAGE-1, using ELISA technique with recombinant CTA or CTA fragments described by Gnjatic et al. [49]. In each assay, sera of patients with known presence or absence of specific reactivity were used as controls. A positive result was defined as extrapolated reciprocal titers >100.

Table 2 Clinical data of non-Hodgkin's lymphoma patients included in ELISA study ($n = 97$)

Clinical data	*n*	%
Age		
≤60	64	66.0
>60	33	34.0
Median age: 54 (17–86)		
Gender		
Male	53	54.6
Female	44	45.4
HIV status		
Negative	92	94.8
Positive	5	5.2
Tumor cell origin		
B-cell	80	82.5
T-cell	16	16.5
Null	1	1.0
Clinical behavior		
Indolent	28	28.9
Aggressive	69	71.1
DLBCL/Non-DLBCL		
Non-DLBCL	47	48.5
DLBCL	50	51.5
Ann Arbor staging		
Early (I or II)	34	35.1
Advanced (III or IV)	62	63.9
Not available	1	1.0
IPI		
Low	29	29.9
Intermediate (low and high intermediate)	48	49.5
High	15	15.5
Not available	5	5.2
Response		
Complete response	41	42.3
Partial response	17	17.5
Stable disease	0	0
Progressive disease	38	39.2
Not available	1	1.0

IPI international prognostic index

Statistical analysis

Associations between the variables were tested by the Pearson χ^2 test (X^2). Mann–Whitney test was used to perform mean comparisons. Overall survival (OS) analyses were performed according to Kaplan–Meier method, and the log-rank was used for analyzing differences between curves. A p value lower than 0.05 was considered as statistically significant.

Results

Tissue microarray (TMA)

Due to the heterogeneous expression pattern of CTAs in most NHL TMA samples, positivity was not graded, and cases were classified as positive for ≥1 CTA (any degree of positivity) or negative (Fig. 1).

Immunohistochemical analyses found that 12 of 106 (11.3 %) NHL samples were positive for at least 1 of 8 antibodies included in our panel. We grouped together the anti-MAGE-A family antibodies—MA454, M3H67, 57B and MAGE-A10#9 due to the overlapping reactivity against several members of MAGE-A family seen in some of these antibodies [41–48]. The most frequently expressed CTA was MAGE-A family (6.6 %), followed by GAGE and NY-ESO-1, which were positive in 5.7 % and 4.7 % of NHL samples, respectively. The 12 CTA-positive cases were: 9/56 DLBCL, 1/2 anaplastic large cell lymphoma, 1/3 lymphoplasmacytic lymphoma and 1/9 peripheral T-cell lymphoma.

We found no statistically significant correlation between CTA positivity and clinical data (Table 3), except for the unexpected finding of higher CTA expression among early stage (19.5 %) compared to advanced stage NHL patients (6.2 %).

The median OS of NHL patients included in our TMA study was 65 months. Survival analysis demonstrated higher median OS among indolent NHL (70 months) compared to aggressive NHL (12 months) ($p = 0.0002$) and worse outcome in DLBCL patients (10 months) compared to the non-DLBCL subgroup (70 months) ($p = 0.0015$). IPI could identify low (median OS not reached), intermediate

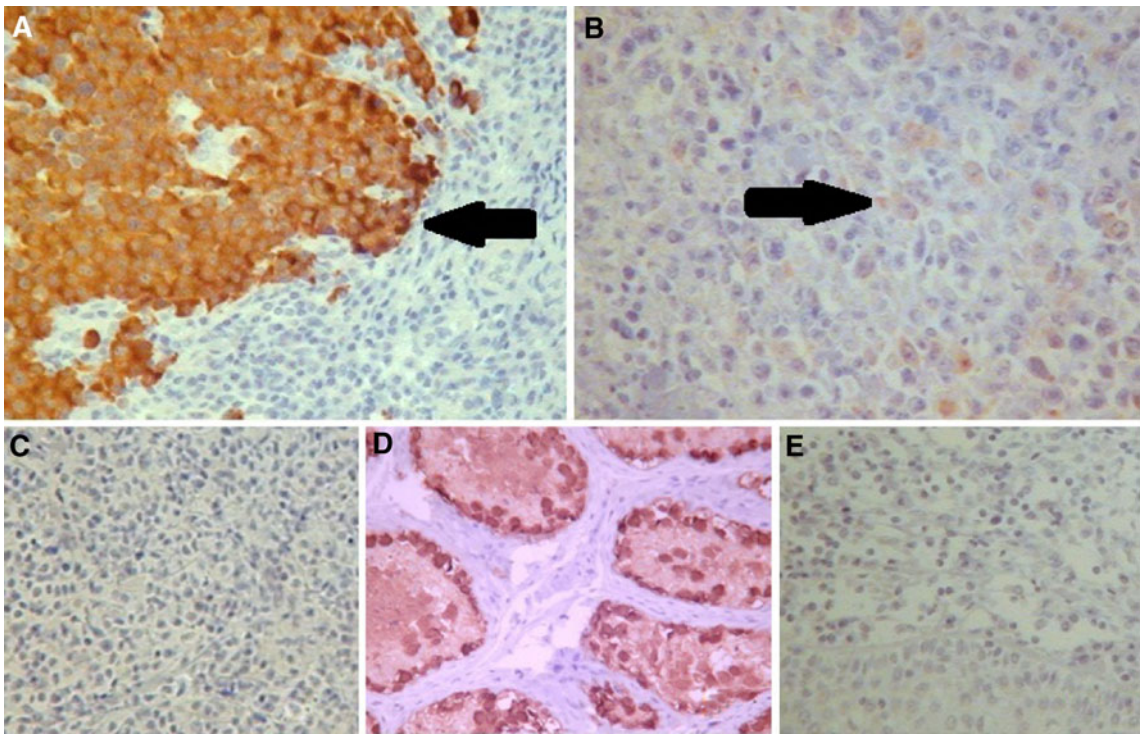

Fig. 1 **a** Illustration of diffuse positivity in tumor representative areas of a lymphoplasmacytic lymphoma sample using MA454 antibody (400×). **b** Illustration of focal positivity in a diffuse large B-cell lymphoma (DLBCL) sample using MA454 antibody (400×). **c** Illustration of a negative DLBCL sample using MA454 antibody (400x). **d** Testis sample used as positive control for MA454 antibody (400×). **e** Tonsils sample used as negative control for MA454 antibody (400×)

(14 months) and high-risk (13 months) patients in our cohort ($p = 0.002$). Despite the difference found in median OS between NHL patients with no CTA expression (65 months) and those who expressed at least one CTA (11 months), it was not statistically significant ($p = 0.0947$). When Cox's multivariate analysis was applied, only IPI ($p < 0.01$) and lymphoma aggressiveness ($p < 0.01$) remained as significant prognostic factors.

Spontaneous humoral immune response (ELISA) in NHL

ELISA assay demonstrated spontaneous humoral immune response against at least 1 CTA from our broad panel in 16 of 97 (16.5 %) NHL serum samples. However, reactivity was low for almost all patients, and strong reactivity (titers >1:1000) was observed in only two DLBCL patients positive for CT45.

The highest positivity was observed against MAGE-A family (8.2 %), when analyzed together. Analyzing individually, CT45 (5.2 %), NY-ESO-1 (5.2 %) and MAGE-A4 (5.2 %) were the most frequent CTAs. Among DLBCL patients, CT45 and NY-ESO-1 were the CTAs most frequently recognized by serum antibodies, with positivity of 8.0 % (3/35) and 6.0 % (2/35), respectively.

Restricting the panel to the 9 CTAs included in TMA, 13 (13.4 %) of 97 NHL sera samples were positive for at least 1 CTA of the panel. MAGE-A4 (4.1 %), MAGE-A3 (3.1 %) and NY-ESO-1 (3.1 %) were the most frequently expressed CTAs.

In the 59 NHL cases with both tissue and sera available, we found 3 positive cases by immunohistochemistry and 10 positive cases by ELISA; 2 of 3 positive cases by immunohistochemistry were also positive by ELISA, and 1 of immunohistochemically positive case was negative by ELISA. The summary of study design and results is illustrated in Fig. 2.

Discussion

To the best of our knowledge, this is the most extensive study performed in this field, evaluating CTA expression in NHL tumor samples, serum immune response analyses, clinical and prognostic data. Analyzing an extensive panel of CTAs, we found an overall low immunohistochemical expression (11.3 %) and serum reactivity against CTAs in our cohort. MAGE-A family (6.6 %), GAGE (5.7 %) and NY-ESO-1 (4.7 %) were the most frequently expressed CTAs. As NHL corresponds to a very heterogeneous

Table 3 Analysis of CTA expression by immunohistochemistry in different non-Hodgkin's lymphoma subgroups ($n = 106$)

Clinical data	*N*	0 CTA (%)	*N*	≥1 CTA (%)	*p* value
NHL cell origin					
B-cell	79	88.8	10	11.2	0.928
T-cell	14	87.5	2	12.5	
Null	1	100.0	0	0	
NHL clinical behavior					
Indolent	31	96.9	1	3.1	0.102
Aggressive	63	85.1	11	14.9	
DLBCL/non-DLBCL					
Non-DLBCL	47	94.0	3	6.0	0.130
DLBCL	47	83.9	9	16.1	
Ann Arbor staging					
I or II	33	80.5	8	19.5	0.046
III or IV	61	93.8	4	6.2	
IPI					
0 or 1	27	87.1	4	12.9	0.990
2 or 3	41	89.1	5	10.9	
4 or 5	17	89.5	2	10.5	
Not available	9	90.0	1	10.0	
Response					
Complete response	41	93.2	3	6.8	0.230
Non-complete response	51	85.0	9	15.0	
Not available	2	100	0	0	

IPI international prognostic index

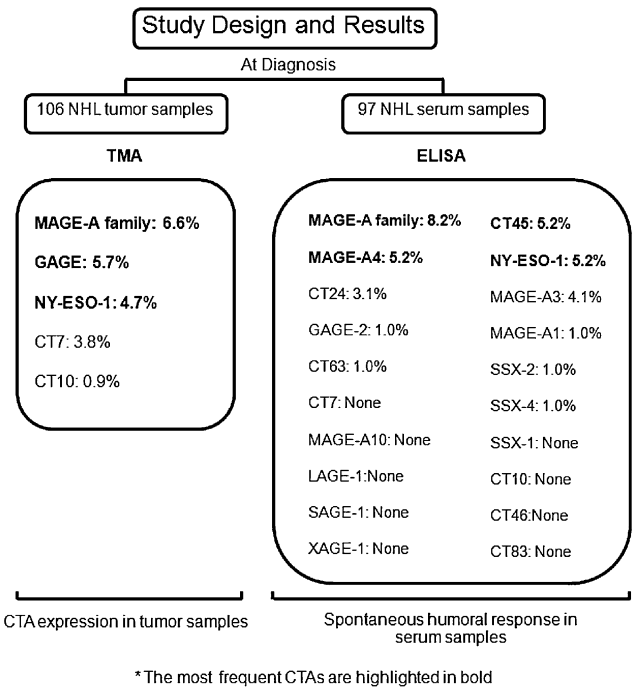

Fig. 2 Summary of study design and results

subtype of tumors, we analyzed separately DLBCL, the most frequent subtype of NHL, where high expression of some CTAs was described in previous studies. However, positivity was equally low to the CTAs included in our panel, and we also did not find statistically significant correlation between CTA expression and prognostic factors or patient outcome. Despite the limited available data about CTA expression in lymphomas to date, our findings are in agreement with most studies performed in this field, using RT-PCR and/or immunohistochemical analysis [33].

Most of studies evaluating CTA expression in different tumors have demonstrated a higher CTA expression in higher histological grade and in advanced stage/metastatic diseases [19, 23–31]. In our study, we found this tendency of CTA expression in indolent versus aggressive NHL and in complete response versus non-complete response subgroup, but differences were not statistically significant. An unexpected finding was the higher positivity in early stage compared to advanced stage disease ($p = 0.046$).

We consider that the poor outcome seen among aggressive NHL patients (most of them DLBCL) in our study probably occurred due to the unavailability of rituximab in Brazilian public hospitals to treat B-cell lymphomas at the time of this study, and the difference in median OS between subgroups according to CTAs positivity likely relates to the known poorer prognosis of aggressive NHL, in whom expression of CTAs was largely confined.

Xie et al. [33] used a CTA panel different from ours. Anti-SCP-1 and commercially available anti-SSX antibodies were not included in our study because SCP-1 is a non-X CTA and both antigens were negative by RT-PCR in a previous study of our collaborating center (LICR—data not published). Among the CTAs studied in both studies (MAGE-A3, MAGE-A4 and CT7), we found lower expression of CT7 in DLBCL (2/56 vs. 2/28) and positive expression of MAGE-A family, which was reported as negative in all DLBCL cases by Xie et al. [33]. The expression of NY-ESO-1 was positive in 4/56 DLBCL of our cohort. Although tested in only 5/28 DLBCL cases, NY-ESO-1 was negative in all samples of Xie's study. Among T-cell lymphomas, we found CTA expression in only 1/16 samples (a peripheral T-cell lymphoma case with positivity for MAGE-A1 and GAGE). Except for SCP-1, that was positive in 9/15 samples, Xie et al. [33] also demonstrated low positivity of other CTAs in T-cell lymphomas.

Even in a partially different cohort, we considered important to assess the serum response against CTAs in NHL because we had a larger panel available for ELISA assay, allowing us to explore the humoral response of NHL patients against not only the CTAs included in immunohistochemical analysis, but also against other CTAs

described as highly expressed in other studies, like CT45 and CT63/PASD1.

Unfortunately, CT45 and CT63/PASD1 were not included in our TMA panel because serological reagents for immunohistochemistry were not available. Although CT63/PASD1 has been described as highly expressed in DLBCL patients by SEREX and T-cell cytotoxic response studies [34–36], in our study, CT63/PASD1 was only tested for serum reactivity in NHL patients, and it was negative in all DLBCL samples. The only CT63/PASD1-positive case was a null-cell phenotype anaplastic large cell lymphoma.

We did not find any plausible explanation to the low expression of CTAs in NHL compared to other malignancies. The findings of global low expression of CTAs in hematological malignancies are in agreement with most of studies in this field published to date. Interestingly, there is high expression of CT7 in multiple myeloma, CT45 in HL and CT63/PASD1 in DLBCL. However, as many new CTAs have been identified recently and some of them have found to be highly expressed in hematological malignancies including NHL (Ex. SP17), it is reasonable to think that other CTAs may be highly expressed in NHL. We should consider that NHL is a very heterogeneous group of tumors, and the CTA expression appears to be very heterogeneous within the various NHL subtypes. As our cohort did not include all subtypes of NHL and in many rare subtypes we analyzed only few samples, we are not able to make any general conclusion about CTA expression in NHL. This is an exploratory study, and further studies accessing the expression of different panels of CTAs in a higher cohort are needed to establish the CTA positivity in NHL patients.

Beside the identification of tumor-specific antigens, optimization of antigen delivery strategies is needed to improve the clinical response to anti-tumoral vaccines. Dendritic cells are considered attractive cancer vaccine platform due to their ability to present peptides derived from tumor-associated antigens on MHC class I, activate tumor-specific cytotoxic T-cells and stimulate the growth and differentiation of B-cells [50, 51]. We believe that an ideal situation for a CTA-based vaccine would be the use of a polyvalent vaccine containing the combination of more frequently expressed CTAs, with an optimized delivery system (DCs) and immunomodulatory agents in a patient treated with effective chemotherapy, presenting only minimal residual disease. Considering the potential benefit of immunotherapy, especially in situations with poor outcome like T-cell lymphoma, mantle cell lymphoma and refractory DLBCL, new studies accessing different panels of CTAs in NHL are needed to establish the role of these antigens as candidates for immunotherapy in NHL patients.

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Conflict of interest The authors do not have any financial or non-financial competing interests for publication of this manuscript.

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