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Title Page

Title

CD40 is a potential marker of favorable prognosis in diffuse large B-cell lymphoma patients treated with immunochemotherapy

Shortened running title

CD40 in diffuse large B-cell lymphoma

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Keywords

Lymphoma, diffuse large B-cell lymphoma, prognostication, CD40

Abstract

We have previously shown that expression of CD40 has a favorable prognostic impact in diffuse large B-cell lymphoma (DLBCL) after anthracycline-based chemotherapy. Here we examined the prognostic value of immunohistochemically defined CD40 in 95 patients with DLBCL treated with both anthracycline-based chemotherapy and rituximab. Using a 10% cut-off level, 77% of the patients had CD40 positive tumors and showed a superior overall survival ($p=0.02$ log-rank, hazard ratio 0.35, 95% CI 0.14-0.88, $p=0.03$ Cox regression). When adjusted for International Prognostic Index in multivariate analysis, CD40 was not an independent prognostic factor (hazard ratio 0.39, 95% CI 0.15-1.04, $p=0.06$ Cox regression). However, even after the introduction of immunochemotherapy, CD40 has a potential prognostic impact in DLBCL. Additional and larger studies are necessary, regarding the immunohistochemical robustness of CD40 and the biological mechanisms that contribute to the superior prognosis in CD40 expressing DLBCL.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common form of malignant lymphoma representing 60-70% of the aggressive lymphomas. The highly variable outcome in patients with DLBCL implies that this entity constitutes a heterogeneous group of neoplasms.

Although almost half of the patients are cured with anthracycline-based chemotherapy and a further improvement is obtained with the addition of rituximab [1], a significant number of patients still die of this disease. There is an obvious need to identify the patients with poor prognosis and allow the development of more risk adapted and targeted treatments.

The International Prognostic Index (IPI) is today the most accepted prognostic model for DLBCL [2]. The subdivision of patients to different risk groups (according to age, Ann Arbor stage, serum lactate dehydrogenase, performance status, and extra nodal sites) is predictive for survival rates and used in treatment stratification. However, patients with identical IPI score show marked variability in survival, suggesting heterogeneity within each risk group.

Consequently, large efforts have been made to identify genetic and molecular markers associated with survival and treatment response. So far, none of the proposed prognostic markers has been stable enough to be incorporated in routine clinical practice.

We have previously demonstrated and confirmed that immunohistochemically defined expression of CD40 in DLBCL results in a favorable prognosis [3,4]. In those studies, the patients were treated with anthracycline-based chemotherapy without rituximab. The aim of the present study was to examine the prognostic impact of CD40 expression in patients treated with the current standard treatment including both anthracycline-based chemotherapy and rituximab. Further, we wanted to evaluate the prognostic impact of different expression levels

of CD40 and if the expression of CD40 was associated with other well-known immunohistochemically defined prognostic markers.

MATERIALS AND METHODS

Patients and Treatment

Clinical data and lymphoma samples were retrospectively collected from 101 patients with de novo DLBCL, stage I-IV, treated during 2002-2006 at the University Hospitals of Helsinki and Lund. All patients were treated with a combination of anthracycline-based chemotherapy and rituximab. Cases with primary CNS involvement, primary mediastinal B-cell lymphoma or transformation from low-grade lymphoma were not included. The protocol of the study was approved by institutional review boards in the units in Finland and Sweden, and the Finnish National Authority for Medicolegal Affairs. Informed consent was not sought, which was decided to be ethically acceptable by the respective institutional review board.

Immunohistochemistry

Pretreatment samples from all patients, taken at the time of diagnosis, had previously been analyzed immunohistochemically for the expression of CD10, BCL6, and MUM1 to determine the germinal center phenotype (GC phenotype), according to the Hans algorithm [5]. In addition, 97 samples were previously analyzed for BCL2 expression, using a cut-off level of 50% positive tumor cells [6].

For immunohistochemical determination of CD40 expression, the paraffin blocks were cut in 4-6 µm thin sections and then dried over night at 60°C and deparaffinised in xylene.

Subsequently the sections were rehydrated through graded alcohol in water and boiled in EDTA buffer (pH 8.9) in microwave oven (800 W for 7 min and 300 W for 15 min). After

boiling, the sections were cooled at room temperature for 20 min and rinsed with water before 5 min placement in a tris-buffered saline. The CD40 antibody (NCL-CD40; Novocastra) was incubated for 25 min in room temperature using a dilution of 1:50. Peroxidase block solution, provided in the EnVision kit, was used to block the endogenous peroxidase for 25 min followed by rinsing the slides with tris-buffered saline. The immunodetection was performed using the Tech- Mate instrument (Dako) and EnVision method (Dako) according to the manufacturer's instructions. The samples were analyzed independently by two hematopathologists (ME and PJ) and disagreements were resolved by joint review using a multiheaded microscope. CD40 expression was estimated according to percent positive stained tumor cells of the total number of tumor cells (0= <10% CD40 positive tumor cells; Figure 1, 1= $\geq 10\%$ CD40 positive tumor cells, 2= $\geq 30\%$ CD40 positive tumor cells; Figure 2, 3= $\geq 90\%$ CD40 positive tumor cells). Normal tonsil tissue was used as a control for staining.

Statistical Analysis

Associations between categorical and/or categorized prognostic factors were evaluated using the chi-square test whereas the log-rank test was used to evaluate differences in overall survival (OS) and progression-free survival (PFS). The Kaplan-Meier method was used to estimate and graphically illustrate the survival rates. To avoid misinterpretation of the unreliable right-hand part of the survival curves, all Kaplan-Meier curves were terminated when less than five patients remained at risk [7]. OS was defined as the time in months from diagnosis until last follow up or death of any reason and PFS as time in months from diagnosis until disease progression, relapse or death of any reason. Uni- and multivariate Cox regression was used to estimate the prognostic effect, hazard ratio (HR), of each factor. All tests were two-sided and the significance level was set to 0.05. SPSS 16.0 (SPSS Inc.

Chicago, IL, USA, 2007) was used for the statistical calculations and Stata 11.0 (StataCorp LP, College Station, TX, USA, 2009) for designing the Kaplan-Meier graphs.

RESULTS

Five patients were excluded because of inadequate lymphoma tissue and one patient because of indeterminate staining. Clinical data from the remaining 95 patients with de novo DLBCL stage I-IV was collected retrospectively. The median age at diagnosis was 64 years with a range from 23 to 84 years. All patients received an anthracycline-based chemotherapy in combination with rituximab. R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) was given to 80 patients, R-CHOEP (R-CHOP with addition of etoposide) was given to 13 patients and other anthracycline-based combination chemotherapy with rituximab was given to two patients. Patients with stage I disease received a minimum of three cycles and patients with stage II-IV disease received at least four cycles. The median follow-up time for the survivors was 38 months. During the follow-up time, 19 patients died and 25 patients had progression/relapse. The five-year OS and PFS for all patients were 72% and 67%.

As expected, the survival for the patients with low IPI scores was significantly (log-rank test) prolonged in comparison to high risk patients (OS 87% vs. 55%, $p=0.01$ and PFS 88% vs. 41%, $p=0.001$). GC phenotype and expression of BCL6 had no impact on OS or PFS, data not shown. Expression of BCL2 was associated with inferior PFS ($p=0.04$), but no significant difference in OS rates was observed ($p=0.24$).

Using cut-off levels of 10%, 30%, and 90% CD40 positive tumor cells, 77%, 63% and 31% of the tumors were considered CD40 positive, respectively. The expression of CD40 using a cut-

off level 10% was not associated with sex or IPI score, neither was it associated with immunohistochemical features such as GC phenotype, BCL2, BCL6, MUM1 or CD10 expression (Table I). Moreover, the higher levels of cut-off for CD40 expression were not associated with the clinical and immunohistochemical features presented in Table I, data not shown.

Expression of CD40 using a cut-off level of 10% was associated with a superior five-year OS as compared to the absence of CD40 expression (OS 77% vs. 54%, $p=0.02$; Figure 3). In univariate analysis using a 10% cut-off level, CD40 showed to be a prognostic factor for OS (HR 0.35, 95% CI 0.14-0.88, $p=0.03$ Cox regression; Table II). When adjusted for IPI in multivariate analysis, CD40 was not an independent prognostic factor for OS (HR 0.39, 95% CI 0.15-1.04, $p=0.06$ Cox regression; Table II). Further, expression of CD40 using a cut-off level of 10% was associated with a superior five-year PFS (73% vs. 49%, $p=0.04$; Figure 4). The univariate analysis showed CD40 to be a prognostic factor for PFS (HR 0.42, 95% CI 0.18-0.98, $p=0.05$ Cox regression; Table III) while no significant prognostic impact of CD40 was shown when adjusted for IPI and BCL2 in multivariate analysis (HR 0.48, 95% CI 0.19-1.25, $p=0.13$ Cox regression; Table III). Considering that neither GC phenotype nor BCL6 expression had any impact on OS and PFS, these variables were not included in multivariate analysis. Similarly, since no significant differences in OS were observed between BCL2 positive and negative groups, we did not include expression of BCL2 in multivariate analysis for OS.

Using a higher cut-off level of 30% CD40 positive tumor cells, no significant difference in OS was observed ($p=0.10$) although this cut-off level was associated with superior PFS ($p=0.03$). Since a 30% cut-off level had no significant prognostic impact on OS, we did not

proceed with univariate or multivariate analysis on this level. An even higher cut-off level of 90% CD40 positive tumor cells was not associated with any prognostic impact on OS ($p=1.0$) or PFS ($p=0.72$).

DISCUSSION

The aim of the present study was to examine the prognostic impact of CD40 in patients with DLBCL after immunochemotherapy. Our results demonstrate a potential prognostic advantage of CD40 expression even after the incorporation of rituximab to lymphoma therapies. The finding is in accordance with our previous observations regarding the prognostic importance of CD40 in DLBCL [3,4] using a cut-off level of 10%, although in the present series, this was not independent of IPI category.

Other immunohistochemical defined prognostic markers such as GC phenotype, BCL2, and BCL6 have shown inconclusive results after the addition of rituximab to chemotherapy [8-11]. In the present study, no significant difference in survival was seen according to GC phenotype or BCL6 status. Expression of BCL2 was associated with inferior PFS but did not significantly affect OS. As the Lunenburg Lymphoma Biomarker Consortium recently demonstrated, lack of harmonization regarding the procedures for immunohistochemistry may contribute to the variation in prognostic impact seen for several markers [12]. The above mentioned studies regarding GC phenotype, BCL2, and BCL6 highlight the need to re-evaluate and search for new prognostic markers as the treatment of DLBCL evolves.

The cell-surface molecule CD40, considered a critical regulator of both humoral and cellular immunity, is a member of the tumor necrosis factor receptor family expressed on all stages of normal B-lymphocytes as well as on dendritic cells, monocytes, epithelial cells, endothelial

cells, and fibroblasts [13]. Moreover, CD40 is expressed in the majority of B-cell malignancies and in 35-100% of solid tumors [14]. CD154, the natural ligand for CD40, is primarily expressed on activated T-helper cells [13]. CD40 activation in B-cells results in proliferation, differentiation, and Ig production [13]. CD40 signaling is also involved in the activation of cytotoxic T-cell response, mostly mediated through enhanced antigen presentation after CD40 interaction between dendritic cells and T-helper cells [15]. Trials with CD40 agonists in B-cell malignancies and solid tumors have shown promising results [16]. A possible mechanism is increased antigen presentation followed by enhanced T-cell response directed against tumor antigens [17], although we observed no correlation between CD40-positivity and high amount of tumor infiltrating T-cells in DLBCL in an earlier study [4].

In normal B-cells and low-grade B-cell lymphoma cell lines, CD40 ligation appears to have a proliferative effect [18]. In contrast, high-grade lymphoma shows a decrease in proliferation both in vitro and in a mouse model after treatment with CD40 ligand [19], indicating disparate response to CD40 stimulation partly depending on the cell type.

A possible mechanism for direct tumor cytotoxicity by CD40 signaling is bax induced apoptosis, as observed in Burkitt lymphoma in vitro after CD40 stimulation [20]. We have previously shown the expression of CD40 and bax to be significantly correlated in patients with DLBCL, [3]. Another pathway induced by CD40 stimulation is the NF-kB mediated induction of transcription factor IRF4 (MUM1), which in turn inhibits the expression of BCL6, a transcriptional repressor essential for B-cell survival [21,22]. This pathway may be disrupted by alterations in the *BCL6* gene as shown in a subset of DLBCL [21]. In the present study, we found no significant association between the protein expression of CD40 and

BCL6, nor did we find any association between CD40 and MUM1. However, this pathway might be of interest for further studies.

In conclusion, we have shown that CD40 is a potential prognostic marker in DLBCL even after the addition of rituximab to standard chemotherapy. The robustness of CD40 as an immunohistochemical prognostic marker needs to be evaluated further in a larger independent data set before the results of this study can be considered more than preliminary and additional studies are necessary to increase the understanding of the biological events connected to CD40 signaling. Immune activation, direct cytotoxicity and possibly the down regulation of the *BCL6* gene are mechanisms that may contribute to the superior prognosis in CD40 expressing DLBCL. Further understanding of the genetic and molecular heterogeneity in DLBCL will allow the development of more tailored treatments and increase the possibilities to achieve cure in a larger number of patients.

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DECLARATION OF INTEREST

The authors reported no potential conflicts of interest.

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TABLES

Table I. Patient characteristics.

Characteristics		Patients, n (%)	CD40 positive¹ n (%)	CD40 negative¹ n (%)	p²
		95 (100)	73 (77)	22(23)	
Sex	male	48 (51)	37(77)	11(23)	0.96
	female	47 (49)	36(77)	11(23)	
IPI	0-2	54 (57)	43(80)	11(20)	0.61
	3-5	36 (38)	27(75)	9(25)	
	missing	5 (5)			
GC ³	GC	44 (46)	32(73)	12(27)	0.38
	non-GC	51 (54)	41(80)	10(20)	
BCL2	pos	59 (62)	44(75)	15(25)	0.47
	neg	32 (34)	26(81)	6(19)	
	missing	4 (4)			
BCL6	pos	50 (53)	35(70)	15(30)	0.08
	neg	41 (43)	35(85)	6(15)	
	missing	4 (4)			
MUM1	pos	44(46)	34(77)	10(23)	0.89
	neg	46(49)	35(76)	11(24)	
	missing	5(5)			
CD10	pos	32(34)	23(72)	9(28)	0.40
	neg	59(62)	47(80)	12(20)	
	missing	4(4)			

¹ 10 % cut-off level.² Chi-square test.³ Germinal center phenotype.

Table II. Univariate and Multivariate Cox regression analysis of OS.

Analysis	Variable	HR	95% CI	p	n
Univariate	CD40 positive vs.negative ¹	0.35	0.14-0.88	0.03	95
	IPI low risk vs. high risk ²	0.30	0.11-0.81	0.02	90
Multivariate	CD40 positive vs.negative	0.39	0.15-1.04	0.06	90
	IPI low risk vs. high risk	0.31	0.12-0.82	0.02	

¹ 10% cut-off level.² IPI low risk includes IPI score 0-2 and IPI high risk includes IPI score 3-5.

Table III. Univariate and Multivariate Cox regression analysis of PFS.

Analysis	Variable	HR	95% CI	P	N
Univariate	CD40 positive vs.negative ¹	0.42	0.18-0.98	0.05	94 ³
	IPI low risk vs. high risk ²	0.21	0.08-0.54	0.001	89
	BCL2 negative vs. positive	0.30	0.09-1.03	0.06	90
Multivariate	CD40 positive vs.negative	0.48	0.19-1.25	0.13	89
	IPI low risk vs. high risk	0.20	0.07-0.55	0.002	
	BCL2 negative vs. positive	0.33	0.10-1.14	0.08	

¹ 10% cut-off level.² IPI low risk includes IPI score 0-2 and IPI high risk includes IPI score 3-5.³ One patient had a PFS of zero months and was excluded from the analysis of PFS.

FIGURES

Figure 1. CD40 staining of DLBCL tumor tissue defined as CD40 negative, with < 10% positive stained tumor cells.

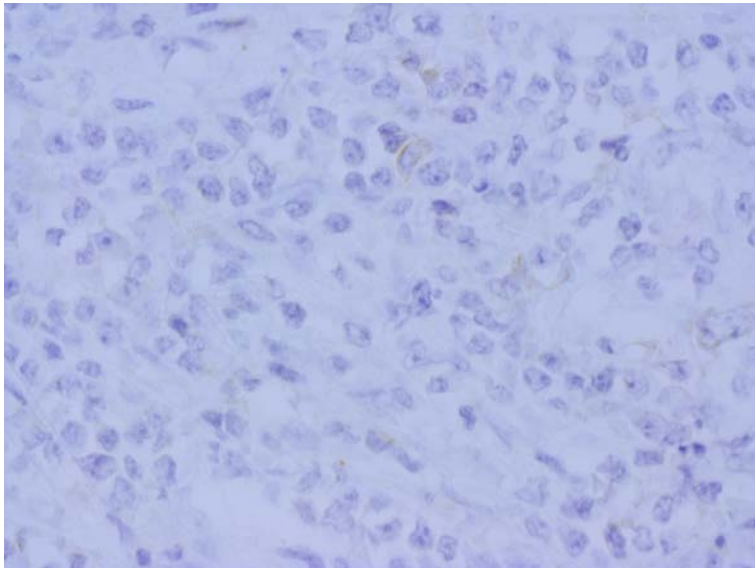


Figure 2. CD40 staining of DLBCL tumor tissue defined as CD40 positive with a cut-off level of 30% positive stained tumor cells.

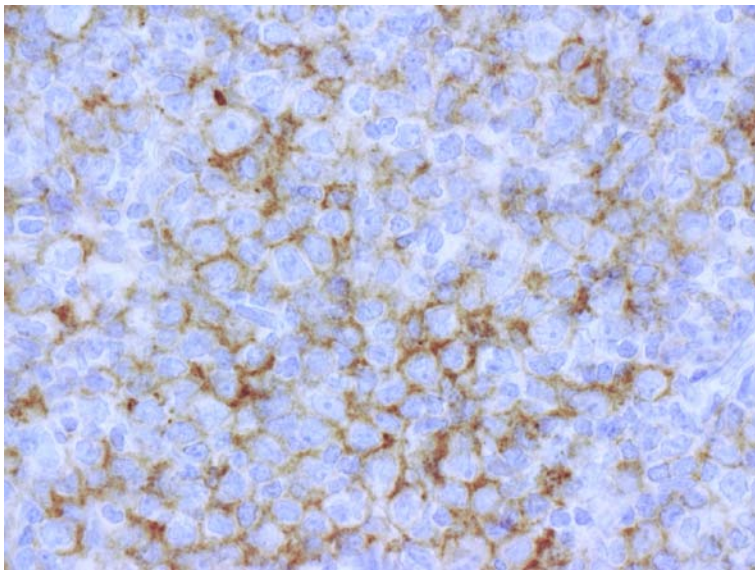


Figure 3. Overall survival stratified by level of CD40 expression using a cut-off level of 10%.

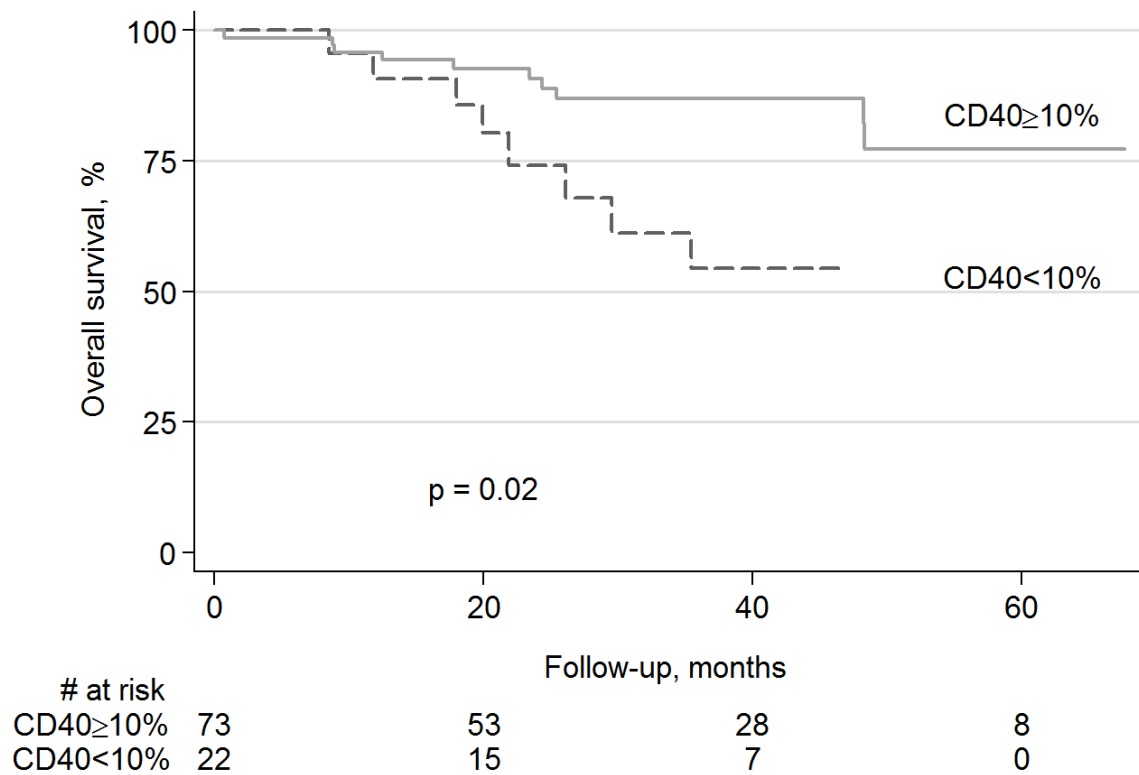


Figure 4. Progression-free survival stratified by level of CD40 expression using a cut-off level of 10%. One patient had a PFS of zero months and was excluded from the analysis.

