

### The significance of gradient expression of chromosome region maintenance protein 1 in large cell lymphoma

Tumor cells depend on nuclear export of macromolecules to sustain their survival.<sup>1</sup> Chromosome region maintenance protein1 (CRM1), encoded by the *XPO1* gene, is the principle receptor mediating the nuclear efflux of proteins.<sup>1</sup> CRM1 (XPO1) is overexpressed in tumor cells to facilitate the increased demand for nuclear export of tumor suppressor proteins, leading to enhanced cell survival.<sup>1-3</sup> The intensity of CRM1 expression has an independent prognostic value in several solid tumors and in acute myeloid leukemia where high-expression was associated with an inferior survival.<sup>2,4</sup> Studies evaluating the presence and degree of CRM1 expression in diffuse large B-cell lymphoma (DLBCL) with respect to prognosis are limited. This topic is important given the recent approval of selinexor, a first-in-class small molecule inhibitor of CRM1, for the treatment of relapsed and/or refractory (R/R) DLBCL without the requirement to demonstrate tumor CRM1 expression.<sup>5</sup> Therefore, we assessed the gradient expression of CRM1 in DLBCL with respect to outcomes in patients treated with anti-CD20 antibody based chemoimmunotherapy.

The study was conducted in accordance with the Declaration of Helsinki following Institutional Review Board approval at Mayo Clinic, Rochester, MN. Patients with DLBCL, primary mediastinal (thymic) large B-cell lymphoma and high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements from the Molecular Epidemiology Resource (MER) cohort at the Mayo Clinic and the University of Iowa were eligible. Paraffin embedded tumor tissue obtained prior to the initiation of treatment from patients who were subsequently treated with chemoimmunotherapy was assessed for CRM1 expression through immunohistochemistry (IHC) on a tissue microarray (TMA) by using a CRM1 monoclonal antibody. Standard slide preparation from paraffin blocks was performed and staining was done in the following order: i) CRM1 (Cell Signaling, catalog-no: 46249, dilution 1:100), ii) MACH 3™ Rabbit Probe HRP Polymer Kit (Biocare Medical, Walnut Creek, CA), iii) R-Polymer HRP: MACH 3™ Rabbit Probe HRP Polymer Kit (Biocare Medical, Walnut Creek, CA, USA), iv) Chromogen: DAB+ (DakoCytomation, Carpinteria, CA, USA), v) two 5-minute incubations with water rinse. Hemotoxylin was used as the counter stain. CRM1 expression by tumor cells was graded based on comparing tumor cell CRM1 staining to background, non-malig-

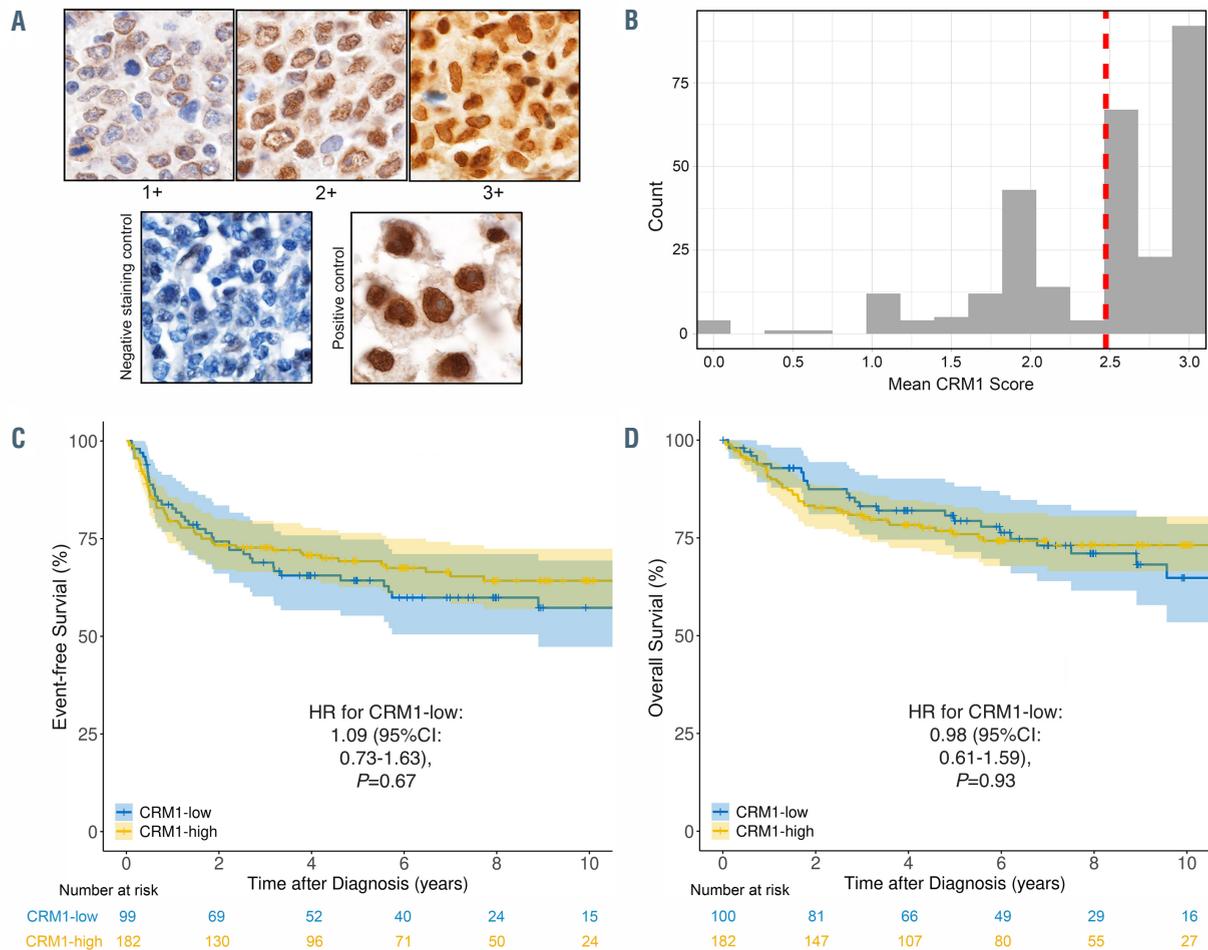


Figure 1. Distribution of the scores of histopathology grading of CRM1 expression on tumor tissue and the prognosis of patients with large-cell lymphoma based on CRM1 protein expression on primary tumor cells. (A) Spectrum of chromosome region maintenance protein1(CRM1) expression on tumor tissue assessed via immunohistochemistry; (B) histogram showing the distribution of the mean expression scores of CRM1, median score for the entire population was 2.5 (vertical red dashed line); (C) event-free survival based on high and low CRM1 expression; (D) overall survival based on high and low CRM1 expression.

**Table 1. Baseline characteristics of patients with large-cell lymphoma based on CRM1 protein expression on primary tumor cells.**

Variable	CRM1-low (N=100)	CRM1-high (N=182)	P
Sex, n, (%)			0.827
Female	42 (42.0)	74 (40.7)	
Male	58 (58.0)	108 (59.3)	
Diagnosis Age			0.239
Median, years, (range)	59.0 (20.0 - 84.0)	61.5 (18.0 - 93.0)	
Subtype, n, (%)			0.7
DLBCL	98 (98.0)	177 (97.3)	
Mediastinal large B-cell	2 (2.0)	5 (2.7)	
Initial Treatment, n, (%)			0.845
MR-CHOP	6 (6.0)	16 (8.8)	
Other IC	4 (4.0)	3 (1.6)	
R-CHOP	66 (66.0)	122 (67.0)	
R-EPOCH	16 (16.0)	23 (12.6)	
R-HYPERCVAD	1 (1.0)	3 (1.6)	
R2-CHOP	6 (6.0)	12 (6.6)	
RAD-RCHOP	1 (1.0)	2 (1.1)	
RCHOP/Zevalin	0 (0.0)	1 (0.5)	
Stage Group n, (%)			0.751
I-II	42 (42.0)	80 (44.0)	
III-IV	58 (58.0)	102 (56.0)	
LDH Group n, (%)			0.454
Missing/unknown	15	17	
≤ Normal n, (%)	47 (55.3)	83 (50.3)	
> Normal n, (%)	38 (44.7)	82 (49.7)	
ECOG PS Group			0.707
< 2	81 (81.0)	144 (79.1)	
≥ 2	19 (19.0)	38 (20.9)	
Num. Extranodal Group n, (%)			0.658
≤ 1	85 (85.0)	151 (83.0)	
> 1	15 (15.0)	31 (17.0)	
IPI n, (%)			0.692
0	17 (17.0)	27 (14.8)	
1	29 (29.0)	43 (23.6)	
2	26 (26.0)	51 (28.0)	
3	19 (19.0)	41 (22.5)	
4	9 (9.0)	17 (9.3)	
5	0 (0.0)	3 (1.6)	
Cell of origin per Hans criteria			0.236
Missing/unknown	28	38	
GCB n, (%)	40 (55.6)	92 (63.9)	
non-GCB n, (%)	32 (44.4)	52 (36.1)	
Cell of origin per Nanostring			0.934
Missing/unknown	52	84	
ABC n, (%)	16 (33.3)	32 (32.7)	
GCB n, (%)	32 (66.7)	66 (67.3)	
MYC Double Hit (FISH) n, (%)			0.617
Missing/unknown	8 (8.0)	10 (5.5)	
Negative	89 (89.0)	164 (90.1)	
Positive	3 (3.0)	8 (4.4)	

ABC: activated B-cell; CRM1: chromosome region maintenance protein1; DLBCL: diffuse large B-cell lymphoma; IPI: international prognostic index; GCB: germinal center B-cell; FISH: fluorescent in-situ hybridization; LDH: lactate dehydrogenase; MR-CHOP: methotrexate, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; RCHOP: rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone; REPOCH: rituximab, etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin; R-HYPERCVAD: rituximab, cyclophosphamide, vincristine, doxorubicin and dexamethasone; R2-CHOP: reulimid, rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone; RAD-RCHOP: radiation with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone.

nant lymphocytes (negative control) and renal cell carcinoma (positive control; known to have a high CRM1 expression).<sup>6</sup> Two expert hematopathologists (RLK, AJW) independently scored CRM1 nuclear staining and assigned a score of 0-3; 0 (no nuclear staining; equal to non-malignant background cells), 1 (dim/weak nuclear staining but greater than background), 2 (moderate nuclear staining with nuclear detail still visible behind the stain) and 3 (strong nuclear staining obscuring most nuclear detail; staining intensity equivalent to renal cell carcinoma), Figure 1A. The average CRM1 score per case across all available cores on the TMA was calculated and the median CRM1 score for the entire cohort was 2.5. Low and high-CRM1 expression were defined as scores of 0-2.4 (CRM1-low) and 2.5-3.0 (CRM1-high), respectively. Scoring reliability between reviewers and between cores was assessed and an intra-class correlation coefficient of 0.75-0.90 was defined as “good scoring reliability”. All time-to-event analyses were conducted from the time of diagnosis. Event-free survival (EFS) was defined as time from diagnosis to progression, retreatment, or death. The association of CRM1 expression and risk of failing to achieve EFS at 24 months after diagnosis (EFS24) was estimated using odds ratios (OR) and 95% Confidence Intervals (CI) from logistic regression models, while the association of CRM1 expression with continuous EFS and overall survival (OS) was estimated using Kaplan-Meier method and hazard ratios (HR) and 95% CI were calculated through Cox regression models.

Eighteen DLBCL TMA with optimal overlap with ongoing sequencing projects were used. After excluding non-chemoimmunotherapy treated patients, tumor tissue cores from 282 patients were analyzed (Table 1). The median age was 61 years (range, 18-93) and 166 (59%) were males. The median follow-up was 88.6 months (95% CI: 82.9-95.5). Quantitative expression of CRM1 was detected in 99% of cases (score of 1 or higher, n=278); therefore the intensity of CRM1 expression was assessed with respect to patient outcomes. One-hundred patients (35%) were categorized in to the CRM1-low intensity cohort; 182 (65%) patients were in the CRM1-high cohort, (Figure 1B). The intra-class correlation coefficient to measure scoring reliability of CRM1 expression was 0.79, meeting the criteria of good reliability between the hematopathologists.

There were no differences in International Prognostic Index (IPI), performance score, lactate dehydrogenase level, age, high-risk disease (double or triple-hit lymphoma), cell-of-origin, or treatment modality at diagnosis between the CRM1-high and the CRM1-low cohorts, (Table 1). The EFS24 was similar in the CRM1-high cohort (30%) compared to CRM1-low cohort (27%), OR 1.09, 95% CI: 0.73-1.63;  $P=0.67$ , (Figure 1C). The OS was not different between cohorts (Figure 1D). The EFS and OS results were similar when adjusted for age, sex, IPI, cell-of-origin, and MYC, BCL2 and BCL6 protein expression and rearrangement status (*data not shown*).

CRM1 is being exploited as a therapeutic target in cancer.<sup>1</sup> The recent United States Food and Drug Administration approval of selinexor for the treatment of R/R DLBCL was based on an overall response rate of 28% with single-agent selinexor suggesting the potential that the intensity level of CRM1 expression may have prognostic significance.<sup>5</sup> In a prior study in DLBCL, the qualitative expression level of CRM1 was an independent negative prognostic marker which associated with higher clinical stage and inferior OS.<sup>7</sup> However, that study was small (n=131), limited by short follow-up (median was not reported; range, 14-65 months), and

heterogeneous treatments with nearly half of the patients receiving chemotherapy instead of the current standard of chemoimmunotherapy.<sup>8</sup> A second study assessed the expression of CRM1 by using a polyclonal-antibody in patients with DLBCL who were treated with chemoimmunotherapy and reported inferior OS associated with high-level of CRM1 expression in activated B-cell or double-expresser types.<sup>9</sup> The median follow-up of the patients was not mentioned in that study and the OS was lower than typical real-world patients with DLBCL treated with chemoimmunotherapy.<sup>10</sup> Importantly, the frequency of CRM1 expression in tumor cells in that study was low at only 40% of cases. This result differs from our study where 99% of cases were CRM1 positive but with variable intensity. The low expression level in that study is surprising given that CRM1/XPO1 is a protein critical for normal cellular function and is expected to be present in all cells to varying degrees.<sup>11</sup> Our study utilized a monoclonal-antibody in contrast to the polyclonal-antibodies used in the two prior studies. In general, monoclonal-antibodies are preferred for clinical use due to their improved specificity for a given epitope.<sup>12</sup> While technical differences in IHC protocols may contribute to different staining outcomes, we hypothesize that CRM1 polyclonal-antibodies may have lower sensitivity in detecting the particular epitope of CRM1 as compared to monoclonal-antibodies, accounting for the differences in positivity rates across DLBCL.

The expression of CRM1 in solid tumors has also been assessed in the past and shown a negative prognostic value with high CRM1 expression.<sup>2</sup> The heterogeneity of tumor biology, patient populations, and treatments may contribute to the disparate findings between solid cancers and ours here in DLBCL. The prevalence of *XPO1* mutation has been documented in DLBCL to be 2-4%, in which the majority were *XPO1*<sup>E571K</sup> mutation.<sup>13,14</sup> In primary mediastinal B-cell lymphoma and Hodgkin lymphoma, the prevalence of *XPO1*<sup>E571K</sup> is close to 25% in some studies.<sup>15</sup> Moreover, the *XPO1*<sup>E571K</sup> mutation has been shown to promote lymphomagenesis and cellular proliferation, alter nuclear cytoplasmic compartmentation of CRM1, and better sensitize cells for CRM1 inhibitors *in vitro* and *in vivo*.<sup>1</sup> The relatively low prevalence of *XPO1* mutation in LCL, and lack of whole exome sequencing (WES) data on our current patient population prevented us from assessing the prognostic significance of *XPO1* mutations in LGL. However, future studies that include WES data on large LCL patient populations may be able to shed further light on this matter.

Strengths of our study include a large dataset, all patients received standard chemoimmunotherapy in a real-world setting, uniform assessment of CRM1 expression using a monoclonal-antibody, independent interpretation by two expert hematopathologists, and long follow-up. We report that CRM1/XPO1 protein is expressed in virtually all DLBCL but the difference in expression intensity did not predict outcomes in patients treated with regimens not containing the CRM1/XPO1-inhibitor, selinexor. Limitations of our study include that, although we have used easily reproducible methods to assess CRM1 expression in tumor cells with good scoring reliability between the two reviewers, the intensity grading algorithm used has not been tested among larger numbers of pathologists. However, based on differences in staining intensities (Figure 1) we demonstrated, there may be potential for this assay to be used in future prospective trials to learn if intensity predicts response to CRM1/XPO1-inhibitor treatment. This issue has not been described in any of the clinical trials which have

used selinexor to-date. The aggressive lymphoma field needs treatment selection factors now more than another marker of overall prognosis. We recommend that CRM1 staining and intensity grading be included in ongoing and future clinical trials to learn if CRM1 intensity predicts selinexor response.

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