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Classical Hodgkin Lymphoma with Reduced β_2M/MHC Class I Expression is Associated with Inferior Outcome Independent of 9p24.1 Status

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Abstract

In classical Hodgkin Lymphoma (cHL), malignant Hodgkin Reed-Sternberg (HRS) cells evade antitumor immunity by multiple mechanisms, including perturbed antigen presentation and enhanced PD-1 signaling. HRS cell expression of the PD-1 ligands is attributable, in part, to copy number alterations of 9p24.1/*CD274(PD-L1)/PDCD1LG2(PD-L2)*. Amplification of *PD-L1/PD-L2* is associated with advanced clinical stage and inferior progression-free survival (PFS) following frontline (induction) therapy. The relationships between altered expression of β_2-microglobulin (β_2M), MHC class I, and MHC class II by HRS cells, *PD-L1/PD-L2* amplification, and clinical outcome in cHL are poorly defined. We assessed these variables in diagnostic biopsy specimens from 108 patients with cHL who were receiving uniform treatment and long-term follow-up, and found decreased/absent expression of β_2M/MHC class I in 79% (85/108) and decreased/absent expression of MHC class II in 67% (72/108) of cases. Patients with decreased/absent β_2M/MHC class I had shorter PFS, independent of *PD-L1/PD-L2* amplification and advanced stage. Decreased or absent MHC class II was unrelated to outcome. These results suggest that MHC class I–mediated antigen presentation by HRS cells is an important component of the biological response to standard chemo/radiotherapy. The paucity of β_2M/MHC class I expression on HRS cells also prompts speculation regarding alternative mechanisms of action of PD-1 blockade in cHL.

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Keywords

Hodgkin Lymphoma; immune escape; antigen presentation; PD-1

Introduction

Primary classical Hodgkin lymphomas (cHLs) are comprised of a mixed infiltrate of inflammatory/immune cells and small numbers of malignant Hodgkin Reed-Sternberg (HRS) cells(1). HRS cells evade antitumor immunity by multiple mechanisms including perturbed antigen presentation and augmented PD-1 signaling that are attributable, in large part, to defined genetic lesions. Recent studies identified HRS cell lines and primary HRS cells with *B2M* mutations that disrupt expression of the β_2-microglobulin (β_2M)/MHC class I dual protein complex at the cell surface(2). Separate studies defined inactivating alterations of the MHC class II transactivator, *CIITA,* in cHL(3). Finally, in virtually all cases of cHL, HRS cells have copy number alterations of 9p24.1, a region that includes *CD274 (PD-L1)* and *PDCD1LG2 (PD-L2)*, and contributes to robust expression of the PD-1 ligands(4).

The biological and clinical significance of the varied immune evasion strategies in cHL are still being elucidated. An initial study on clinical samples from patients with cHL suggested that loss of β_2M protein was, paradoxically, associated with improved clinical outcome(2). In contrast, our recent analysis of diagnostic biopsy samples from patients treated with Stanford V, a standard induction regimen comparable to ABVD (adriamycin, bleomycin, vinblastine and dacarbazine), revealed highly significant associations between amplification of *PD-L1/PD-L2* and advanced stage disease at presentation and inferior progression-free survival (PFS)(4). The genetic basis of PD-1–mediated immune evasion likely explains the efficacy of PD-1 blockade in cHL. In pilot studies and confirmatory phase II trials, patients with relapsed/refractory cHL treated with PD-1 blocking antibodies had response rates of 65–87% and long-lasting remissions(5–7).

We sought to clarify the prognostic significance of perturbed MHC class I and MHC class II antigen presentation by HRS cells and explore the relationship between antigen presentation and 9p24.1 genetic alterations in cHL. Herein, we characterize cell surface β_2M, MHC class I, and MHC class II expression in a series of uniformly treated cHL patients with long-term follow-up and defined 9p24.1/*PD-L1/PD-L2* alterations (4).

Materials and Methods

Patient samples and 9p24.1 genetic analyses

The samples used in this study were from a previously described 108 patient series, with institutional review board approval (4). Formalin-fixed paraffin-embedded (FFPE) tumor samples and select pathological and clinical data from 108 patients with newly diagnosed cHL, treated with the Stanford V chemotherapy regimen + modified involved field radiation (IFR), were obtained from Stanford University(4). Median patient follow-up was 9 years. In this series, 9p24.1/*PD-L1*/*PD-L2* alterations were characterized by fluorescence *in situ* hybridization (FISH) as previously described(4,8).

Immunohistochemistry

Immunohistochemical staining (IHC) for β_2M (Dako, A0072, 1:6000), MHC class I (Abcam, EMR8-5, 1:6000) and MHC class II (Dako, CR3/43 M0775, 1:750) was performed using an automated staining system (Bond III, Leica Biosystems, Buffalo Grove, IL) according to the manufacturer's protocol following antigen retrieval (Bond, ER2 solution). Hematoxylin counterstain was subsequently applied.

Scoring Stained Tissue Sections

Staining on two or more adjacent HRS cells was used to determine membrane expression of the antigen presentation proteins, β_2M, MHC class I, and MHC class II. HRS cells were evaluated for the presence of positive membrane staining of each biomarker. If present, the relative intensity of HRS cell membrane expression, relative to adjacent non-malignant inflammatory cells, was determined. β_2M, MHC class I, and MHC class II IHC were optimized on a test series of cHLs. In a subset of cases, β_2M, MHC class I, and/or MHC class II expression on the vast majority of HRS membranes was equivalent to or greater than that observed on adjacent, nonmalignant inflammatory cells (Supplementary Fig. S1, case 1 for β_2M and MHC class I, case 2 for MHC class II). In a subset, no β_2M, MHC class I, and/or MHC class II expression was detected on the vast majority of HRS cells or cell membranes, despite appropriate internal controls (Supplementary Fig. S1, case 3 for β_2M and MHC class I, case 1 for MHC class II). An additional subset of cases exhibited heterogeneous HRS cell staining, including HRS cells with unequivocally positive but reduced membrane staining, relative to adjacent non-malignant cells, and those with a combination of reduced and complete loss of staining in a subset of cells (Supplementary Fig. S1, case 2 for β_2M and MHC class I, case 3 for MHC class II).

We devised a 3-tiered scoring system to categorize the predominant patterns of β2M, MHC class I, and MHC class II expression by HRS cells in each case. For cases categorized as *positive*, at least 90% of evaluable HRS cells showed positive membrane staining for the biomarker at levels equivalent to, or greater than, that of adjacent nonmalignant inflammatory cells. For cases categorized as *negative*, at least 90% of evaluable HRS cells showed no detectable membrane staining for the biomarker relative to nonmalignant inflammatory cells. For cases categorized as *decreased*, positive membrane staining of HRS cells was present and unequivocally reduced relative to surrounding cells and/or positive staining was observed in less than 90% of evaluable HRS cells.

Stained slides from the clinical series were scored separately for each of the markers by two independent hematopathologists (SR; GP), blinded to the clinical data. *Kappa* statistics were then generated on the two sets of independent scores (Supplementary Table 1). Cases with scores that were found to be discordant between the two independent reviewers were reconciled in a consensus conference. The consensus score was used for the final analysis.

Statistical Analysis

Progression-free survival (PFS) - time from diagnosis until first progression or death, censored at time last known alive and progression-free - was determined by method of Kaplan and Meier and compared using log-rank tests. PFS is a preferred metric of response

to frontline (induction) therapy, rather than overall survival, as patients who fail induction therapy routinely undergo salvage high-dose chemotherapy and stem cell rescue—a treatment that is curative in a subset of patients. Proportional hazards (PH) regression with Firth's penalized likelihood were fit using disease stage, *PD-L1/PD-L2* amplification, and β_2M, MHC class I, and MHC class II expression. Cox PH models were compared using likelihood ratio tests, and Wald *P* values were reported for covariates. Associations between continuous, nominal, and ordinal variables were assessed using Wilcoxon rank-sum, Fisher's exact, and Kruskal-Wallis tests, respectively. All *P* values were two-sided; values ≤ 0.05 were considered statistically significant.

Results

Expression of β_2M, MHC class I, and MHC class II

We optimized the immunohistochemical staining and scoring methods for β2M, MHC class I and MHC class II on a small series of FFPE diagnostic tissue biopsies from patients with cHL (Supplementary Fig. S1). We next evaluated HRS cell expression of β_2M, MHC class I, and MHC class II in diagnostic biopsy specimens from 108 patients with cHL who received uniform treatment and had long term follow-up (median: 9 years) and previously characterized 9p24.1/*PD-L1/PD-L2* alterations(4). Each cHL was classified as *positive*, *decreased*, *negative*, or *not assessable* for HRS cell membrane expression of β_2M, MHC class I and MHC class II. In each case, the HRS cell membrane expression was compared to that of non-malignant cells within the same tissue section. Representative examples are shown in Fig. 1A. Cases included those with positive HRS cell membrane expression of β_2M, MHC class I and MHC class II (case 1, Fig. 1A); decreased HRS cell membrane expression of β_2M, MHC class I and MHC class II (case 2, Fig. 1A); and no HRS cell membrane expression of the 3 markers (case 3, Fig. 1A). Additional cases were positive for β_2M and MHC class I and negative for MHC class II (case 4, Fig. 1A), or negative for β_2M and MHC class I but positive for MHC class II (case 5, Fig. 1A).

The patterns of β_2M, MHC class I, and MHC class II expression in the cHL series are summarized in Fig. 1B. For β_2M, HRS cells were positive in 16% (17/108), decreased in 27% (29/108), negative in 53% (57/108), and unevaluable in 5% (5/108) of cases (Fig. 1B). For MHC class I, HRS cells were positive in 18% (19/108), decreased in 31% (34/108), negative in 47% (51/108), and unevaluable in 4% (4/108) of cases (Fig. 1B). The association between categories of cell surface β_2M and MHC class I expression (positive, decreased, or absent) by HRS cells was highly significant across cases (Fig. 1B; $P < 0.001$), suggesting that *B2M* alterations might be a structural basis for MHC class I loss(2). Overall, HRS cells in less than 20% of the cases showed positive membrane β_2M or MHC class I staining.

MHC class II expression on HRS cells was positive in 31% (34/108), decreased in 37% (40/108), negative in 30% (32/108), and unevaluable in 2% (2/108) of cases (Fig. 1B). Among the cHLs that were positive for cell surface MHC class II, only 12% (4/34 cases) were also positive for β_2M/MHC class I; the remainder had decreased/negative β_2M/MHC class I expression (88% [30/34 cases], Fig. 1B). Consistent with these findings, MHC class II expression was not associated with either β_2M or MHC class I expression (Fig. 1B; $P = 0.47$ and $P = 0.21$, respectively).

Clinical and biological factors and MHC class I and II expression

We next examined the relationship between specific clinical features and β_2M, MHC class I, and MHC class II HRS cell expression in the patients in whom all three antigen presentation pathway components were evaluable ($n = 103$, Table 1). In this series, there was no significant association between age, stage, B symptoms or bulky disease and altered β_2M, MHC class I, or MHC class II expression (Table 1). In contrast, there were significant associations between the mixed cellularity cHL (MCHL) subtype and positive β_2M and MHC class I expression, and between EBV$^+$ cHL and positive β_2M and MHC class I expression ($P < 0.001$, Table 1), consistent with previous reports(9–12).

Association of outcome with β_2M, MHC class I and MHC class II expression

We next evaluated a potential association between β_2M, MHC class I and MHC class II expression and outcome (PFS) in this series of uniformly treated cHL patients with long-term follow-up (Fig. 2). PFS was comparable in patients whose HRS cells had decreased or negative β_2M expression (Fig. 2A, left panel). However, patients whose HRS cells had decreased/negative β_2M expression had significantly shorter PFS than those whose HRS cells were positive for β_2M ($P = 0.037$, Fig. 2B, left panel). Similarly, PFS was comparable in patients with decreased or negative MHC class I expression in HRS cells (Fig. 2A, middle panel) but significantly shorter than that of patients with positive MHC class I expression in HRS cells ($P = 0.031$, Fig. 2B, middle panel). Consistent with these results, reduced (decreased/negative) expression of β_2M and MHC class I had adverse prognostic significance in univariate models (Table 2, $P = 0.02$). MHC class II expression by HRS cells was not significantly associated with PFS in these patients ($P = 0.60$, Fig. 2 and Table 2). In addition, neither the cHL subtype (MCHL vs. nodular sclerosis HL) nor EBV status was associated with PFS (not shown).

Antigen presentation pathway components and 9p24.1/*PD-L1/PD-L2* status

In this series of cHL patients, we found that 9p24.1/*PD-L1*/*PD-L2* amplification was associated with advanced stage disease and inferior PFS(4). Given these findings, we next evaluated the prognostic significance of *PD-L1/PD-L2* amplification in patients with defined HRS cell expression of β_2M/MHC class I (Fig. 2C). In patients with positive HRS cell expression of β_2M and MHC class I, PFS was not affected by 9p24.1 amplification, although only 3 patients had 9p24.1 amplification and positive β2M and MHC class I (Fig. 2C left and middle panels). In marked contrast, 9p24.1 amplification adversely impacted PFS in patients whose HRS cells had decreased/negative β_2M/MHC class I expression (Fig. 2C, left and middle panels). Importantly, these results were highly significant despite the small numbers of 9p24.1, β_2M-positive, and MHC class I-positive patients ($P = 0.014$ and $P = 0.013$, respectively).

In multivariable models of 1) decreased/negative MHC class I expression and 9p24.1 amplification or, 2) decreased/negative MHC class I expression and advanced stage disease, both features retained adverse prognostic significance (Table 2). When all three features were included in the multivariable model, decreased/negative expression of MHC class I still retained adverse prognostic significance (Table 2, $P = 0.05$). Similar results were obtained in Cox models containing β_2M rather than MHC class I (Supplementary Table S2).

Discussion

In this analysis, we found that: (i) decreased or absent β_2M and MHC class I expression and decreased or absent MHC class II expression on HRS cells occurs in approximately 80% and 70% of cHL cases, respectively; (ii) decreased/absent β_2M/MHC class I expression, but not decreased/absent MHC class II expression, is associated with shorter PFS; and (iii) the prognostic value of decreased/absent β_2M/MHC class I expression is independent of *PD-L1/PD-L2* amplification and advanced stage disease.

The *B2M* subunit is required for assembly of MHC class I on the cell surface of nucleated cells. As expected, we observed a high concordance between β_2M and MHC class I IHC scores. For cases of cHL categorized as negative for β_2M expression, we found that loss of β_2M from the HRS cells was complete. In contrast, HRS cells in certain cases exhibited cytoplasmic expression of MHC class I in the absence of membranous staining (Fig. 1, cases #3 and #5 vs. case #4). These data suggest that loss of β_2M expression is the predominant mechanism for deficient cell surface MHC class I protein expression in cHL, as previously suggested by genetic studies(2).

In contrast to prior reports in cHL, we observed unequivocally reduced, but not completely absent, β_2M, MHC class I, and class II membrane expression on HRS cells relative to normal, surrounding inflammatory cells in a significant subset (30–40%) of cases(2,13). We used a 3-tiered scoring system—positive, decreased, and negative—to capture the heterogeneity of β_2M/MHC class I and MHC class II protein expression. Cases with reduced, but not completely absent, expression of the β_2M/MHC class I, and MHC class II proteins may have single-allele loss or a heterozygous inactivating mutation in genes directly encoding these proteins (such as single-allele inactivation of *B2M* or single-copy loss of the HLA locus) or alterations in genes encoding critical transcriptional regulators (such as *NLRC5* and *CIITA*)(3,14,15). We also observed cases, classified as *decreased*, with positive expression in only a subset of HRS cells, suggesting that subclones with distinct characteristics can exist within a single case.

In contrast to a recent study(2), we find that cHL patients with decreased or negative β_2M expression have inferior outcomes (Fig. 2 and Table 2). Unlike the prior study, patients in this series received uniform treatment at a single institution and had pre-specified clinical follow-up for a median of 9 years. In further contrast, this series includes a representative mix of early and advanced stage disease and nodular sclerosis HL (NSHL) and MCHL. As previously reported for cHL, we found that patients with advanced stage disease had inferior PFS, and patients with MCHL and/or EBV$^+$ cHL had significantly higher β_2M/MHC class I expression on HRS cells(4,9–12). Given these characteristics, we believe that the current series provides a comprehensive framework for analyzing the prognostic significance of β_2M, MHC class I, and MHC class II expression in newly diagnosed cHL patients treated with standard induction therapy. In addition, these data highlight the likely biological importance MHC class I–mediated antigen presentation by HRS cells to cytotoxic T cells for optimal clinical response to nonimmune therapy. That MHC class I expression is associated with a more favorable outcome to standard therapy in cHL is consistent with findings in multiple other tumor types(16–19).

The paucity of β_2M/MHC class I expression on HRS cells also prompts speculation regarding additional mechanisms of action of PD-1 blockade in cHL. In other tumor types, clinical responses to standard chemotherapy and immunotherapies, including checkpoint blockade, have been associated with the presence of $CD8^+$ T cells within the tumor microenvironment, underscoring the importance of β_2M/MHC class I–mediated antigen presentation by malignant cells(16,20–22). Patients with relapsed/refractory cHL who are treated with PD-1 blocking antibodies have response rates of 65–87% and long-lasting remissions. Yet, the complete loss of MHC class I and β_2M expression by HRS cells in approximately one-half of cHLs suggests that $CD8^+$ cytotoxic T cell–mediated killing of HRS cells may not be the only mechanism of antitumor immunity augmented by PD-1 blockade. Given our findings, the significance of impaired antigen presentation by HRS cells on the quality and durability of clinical responses to PD-1 inhibitors will be of great interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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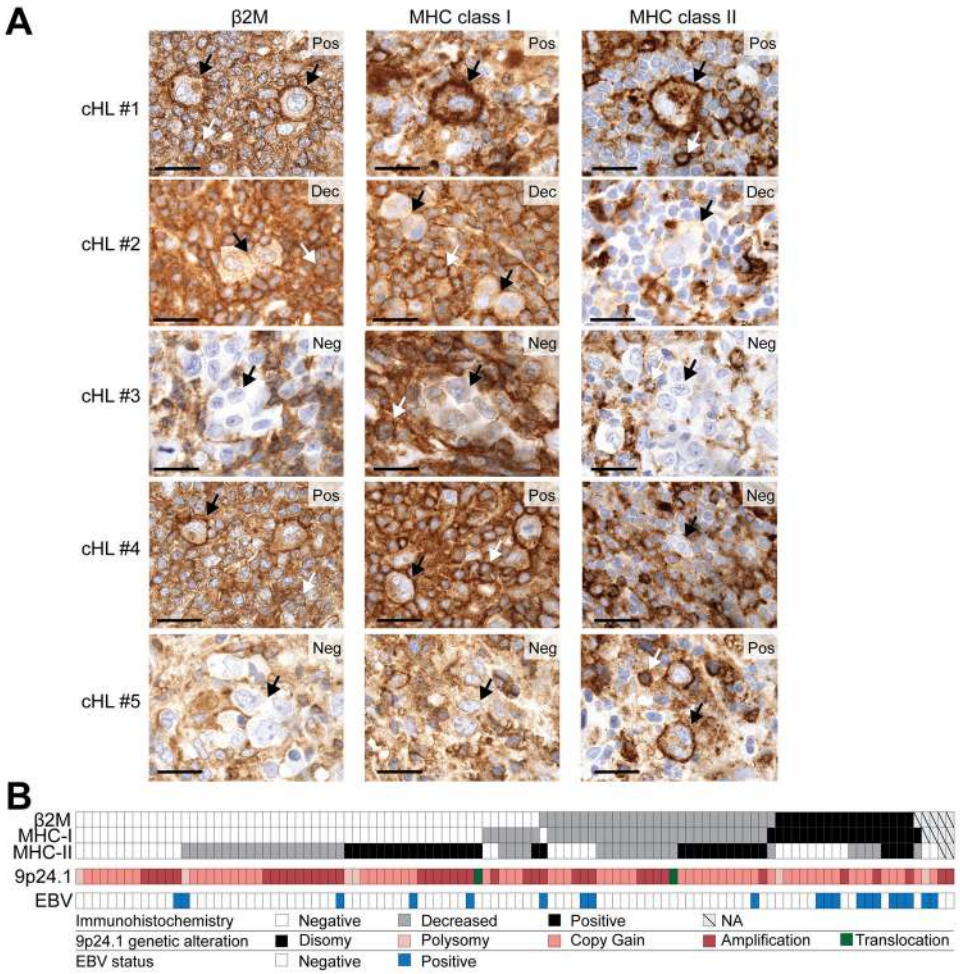

Figure 1. β_2M, MHC class I, and MHC class II expression in cHL patients

(A) β_2M, MHC class I and MHC class II immunohistochemical staining in 5 representative cHL patients: #1, positive for all markers (Pos); #2, decreased for all markers (Dec); #3, negative for all markers (Neg); #4, positive for β_2M and MHC class I, negative for MHC class II and #5: negative for β_2M and MHC class I, positive for MHC class II. Individual HRS cells are depicted with a black arrow. The white arrows indicate expression on surrounding, non-malignant inflammatory cells. Scale bar, 50 μm. (B) Heatmap representing the distribution of β_2M, MHC class I (MHC-I) and MHC class II (MHC-II) expression in the 108 cHL patients. White = negative, grey = decreased, black = positive and hatched = not assessable (NA). 9p24.1 genetic alterations, light pink = polysomy, medium red = copy gain, dark red = amplification and EBV status, white = negative, blue = positive, are depicted below.

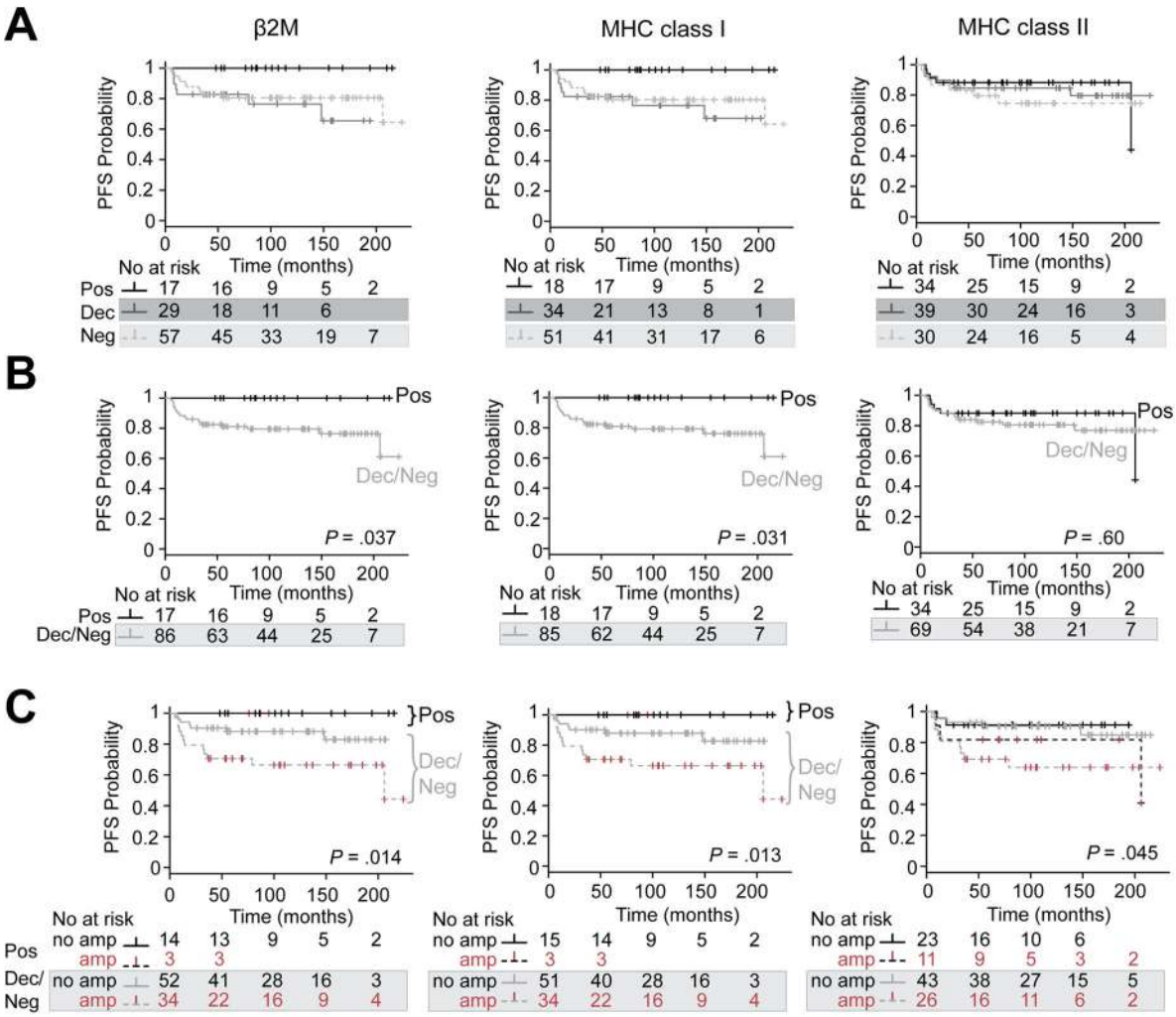

Figure 2. PFS by β_2M, MHC class I, and MHC class II expression in the cHL cohort

(A) PFS for patients with positive (Pos), decreased (Dec) and negative (Neg) HRS cell membrane expression of β_2M (left panel), MHC class I (middle panel) and MHC class II (right panel). (B) PFS for patients with positive vs. decreased/negative HRS cell expression of β_2M (left panel), MHC class I (middle panel) and MHC class II (right panel). (C) PFS for patients whose HRS cells have positive or decreased/negative β_2M (left panel), MHC class I (middle panel) and MHC class II (right panel) expression in the presence or absence of *PD-L1/PD-L2* amplification.

Table 1

Clinical Factors

		All	β2M			MHC Class I			MHC Class II		
Characteristic		(n=103)	Dec/Neg (n=86)	Pos (n=17)	*P*	Dec/Neg (n=85)	Pos (n=18)	*P*	Dec/Neg (n=69)	Pos (n=34)	*P*
Age - yr	Median (range)	30 (18–69)	29 (18–69)	35 (19–66)	0.13	29 (18–69)	36 (19–66)	0.10	29 (18–69)	31 (18–62)	0.74
Males	n (%)	45	34 (76)	11 (24)	0.07	33 (73)	12 (27)	0.04	31 (69)	14 (31)	0.83
Stage	n (%)				0.18			0.32			0.45
ES-F		31	23 (74)	8 (26)		23 (74)	8 (26)		21 (68)	10 (32)	
ES-U		39	34 (87)	5 (13)		34 (87)	5 (13)		23 (59)	16 (41)	
AS		33	29 (88)	4 (12)		28 (85)	6 (15)		25 (76)	8 (24)	
B symptoms	n (%)	37	33 (89)	4 (11)	0.28	32 (86)	5 (14)	0.59	26 (70)	11 (30)	0.67
Bulky disease	n (%)	48	42 (87)	6 (13)	0.43	42 (87)	6 (13)	0.30	32 (67)	16 (33)	>0.99
Histologic subtype	n (%)				*<0.001*			*<0.001*			0.03
Nodular sclerosis		91	81 (89)	10 (11)		80 (88)	11 (12)		59 (65)	32 (35)	
Mixed cellularity		9	3 (33)	6 (67)		3 (33)	6 (67)		9 (100)	0 (0)	
cHL - nos		3	2 (67)	1 (33)		2 (67)	1 (33)		1 (33)	2 (67)	
EBV	n (%)				*<0.001*			*<0.001*			0.59
Negative		85	77 (91)	8 (9)		76 (89)	9 (11)		58 (68)	27 (32)	
Positive		18	9 (50)	9 (50)		9 (50)	9 (50)		11 (61)	7 (39)	

ES-F: early-stage favorable; ES-U: early-stage unfavorable; AS: advanced stage; cHL - nos: classical Hodgkin lymphoma - not otherwise specified

Table 2

Outcome (progression-free survival) - Cox univariate and multivariable models

Univariate models	**HR**	**Std. error**	***P***
β2M			
β2M decreased/negative	9.2	1.47	0.02
MHC class I			
MHC class I decreased/negative	9.7	1.47	0.02
MHC class II			
MHC class II decreased/negative	1.2	0.52	0.66
9p24.1 genetic status			
9p24.1 amplification	3.3	0.47	0.01
Clinical factors			
Advanced stage	3.1	0.46	0.01
Multivariable models	**HR**	**Std. error**	***P***
MHC class I decreased/negative, 9p24.1 amplification			
MHC class I decreased/negative	7.3	1.48	0.05
9p24.1 amplification	2.7	0.47	0.03
MHC class I decreased/negative, advanced stage			
MHC class I decreased/negative	8.9	1.47	0.03
Advanced stage	2.9	0.47	0.02
MHC class I decreased/negative, advanced stage, 9p24.1 amplification			
MHC class I decreased/negative	7.4	1.48	0.05
Advanced stage	2.5	0.47	0.04
9p24.1 amplification	2.3	0.48	0.07