Published Ahead of Print on April 11, 2016 as 10.1200/JCO.2016.66.4482
The latest version is at http://jco.ascopubs.org/cgi/doi/10.1200/JCO.2016.66.4482

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

PD-L1 and *PD-L2* Genetic Alterations Define Classical Hodgkin Lymphoma and Predict Outcome

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Published online ahead of print at www.jco.org on April 11, 2016.

Processed as a Rapid Communication manuscript.

Supported by National Institutes of Health Grant No. R01 CA161026 and the Miller Family Fund (M.A.S.).

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Presented at the 57th American Society of Hematology Annual Meeting, Orlando, FL, December 4-8, 2015.

Authors' disclosures of potential conflicts of interest are found in the article online at www.jco.org. Author contributions are found at the end of this article.

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0732-183X/16/3499-1/$20.00

DOI: 10.1200/JCO.2016.66.4482

ABSTRACT

Purpose
Classical Hodgkin lymphomas (cHLs) include small numbers of malignant Reed-Sternberg cells within an extensive but ineffective inflammatory/immune cell infiltrate. In cHL, chromosome 9p24.1/*PD-L1/PD-L2* alterations increase the abundance of the PD-1 ligands, PD-L1 and PD-L2, and their further induction through Janus kinase 2–signal transducers and activators of transcription signaling. The unique composition of cHL limits its analysis with high-throughput genomic assays. Therefore, the precise incidence, nature, and prognostic significance of *PD-L1*/*PD-L2* alterations in cHL remain undefined.

Methods
We used a fluorescent in situ hybridization assay to evaluate *CD274/PD-L1* and *PDCD1LG2/PD-L2* alterations in 108 biopsy specimens from patients with newly diagnosed cHL who were treated with the Stanford V regimen and had long-term follow-up. In each case, the frequency and magnitude of 9p24.1 alterations—polysomy, copy gain, and amplification—were determined, and the expression of PD-L1 and PD-L2 was evaluated by immunohistochemistry. We also assessed the association of 9p24.1 alterations with clinical parameters, which included stage (early stage I/II favorable risk, early stage unfavorable risk, advanced stage [AS] III/IV) and progression-free survival (PFS).

Results
Ninety-seven percent of all evaluated cHLs had concordant alterations of the *PD-L1* and *PD-L2* loci (polysomy, 5% [five of 108]; copy gain, 56% [61 of 108]; amplification, 36% [39 of 108]). There was an association between PD-L1 protein expression and relative genetic alterations in this series. PFS was significantly shorter for patients with 9p24.1 amplification, and the incidence of 9p24.1 amplification was increased in patients with AS cHL.

Conclusion
PD-L1/PD-L2 alterations are a defining feature of cHL. Amplification of 9p24.1 is more common in patients with AS disease and associated with shorter PFS in this series. Further analyses of 9p24.1 alterations in patients treated with standard cHL induction regimens or checkpoint blockade are warranted.

J Clin Oncol 34. © 2016 by American Society of Clinical Oncology

INTRODUCTION

Patients with newly diagnosed classical Hodgkin lymphoma (cHL) are currently treated with empirical combination chemotherapy regimens, such as ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine). An alternative combined modality regimen, Stanford V (doxorubicin, vinblastine, mechlorethamine, vincristine, bleomycin, etoposide, prednisone) and modified involved field radiation, is equally effective.[1-3] Although many patients respond well to these regimens, 20% to 30% experience a relapse after treatment or fail to respond to induction therapy.[4,5] For these patients, new therapies that are based on the unique biology of cHL are urgently needed.

Primary cHLs include small numbers of malignant Reed-Sternberg (RS) cells surrounded by an extensive but ineffective inflammatory/immune cell infiltrate.[6-8] In cHL, chromosome 9p24.1/*CD274(PD-L1)/PDCD1LG2(PD-L2)* alterations have been shown to increase the abundance of these PD-1 ligands. The 9p24.1 amplicon also contains *JAK2*, and copy number–dependent Janus kinase 2–signal transducers and activators of

transcription (JAK2-STAT) signaling further increases PD-1 ligand expression.[6] Less frequent chromosomal rearrangements of the PD-1 ligand loci have also been described.[9] Furthermore, Epstein-Barr virus (EBV) infection can increase expression of PD-1 ligands in EBV-positive Hodgkin lymphomas (HLs).[10]

PD-1 ligands engage the PD-1 receptor on T cells and induce PD-1 signaling and T-cell exhaustion by reversible inhibition of T-cell activation and proliferation.[11] Tumor cells expressing PD-1 ligands on their surface use the PD-1 pathway to evade an effective antitumor immune response.[12]

The genetic bases of PD-1 ligand deregulation and overexpression in cHL suggest the potential vulnerability of cHL to PD-1 blockade. For this reason, PD-1 blockade with nivolumab was evaluated in a phase I/Ib study of 23 heavily pretreated patients with relapsed/refractory cHL. In this pilot study, the overall response rate was 87%, and the median duration of response was not reached at 88 weeks.[13,14] All 10 evaluable patients had *PD-L1*/*PD-L2* copy number alterations (CNAs), increased expression of the PD-1 ligands, and active JAK-STAT signaling.[13] In another phase Ib study of relapsed/refractory cHL, PD-1 blockade with pembrolizumab resulted in an overall response rate of 65% without serious adverse events.[15] These pilot studies showed that PD-1 blocking agents were well tolerated in relapsed/refractory cHL and associated with high response rates and long-lasting remissions.[13-15]

The unique cellular composition of primary cHL limits its analysis with high-throughput genomic assays. Therefore, the precise incidence, nature, and prognostic significance of *PD-L1* and *PD-L2* alterations in cHL remain undefined. We use a recently developed fluorescent in situ hybridization (FISH) assay to characterize 9p24.1/*PD-L1*/*PD-L2* alterations in a cohort of 108 patients with newly diagnosed cHL who were treated with the Stanford V regimen and have long-term outcome data.

METHODS

Patients

This study was an institutional review board–approved collaborative effort among Stanford University, Brigham and Women's Hospital, VU University Medical Center, and the Dana-Farber Cancer Institute. Formalin-fixed paraffin-embedded (FFPE) tumor samples and clinical data from 108 patients with newly diagnosed cHL were obtained from Stanford University. The pathology on all cases was reviewed and diagnoses confirmed independently by two expert hematopathologists (Y.N. and S.J.R.). Study patients were treated on three concurrent clinical protocols of the Stanford V chemotherapy regimen plus modified involved field radiation (IFR) for clinically defined risk groups as previously described.[16,17] Patients with Ann Arbor early stage (I/II) nonbulky disease and no B symptoms were treated with 8 weeks Stanford V and 30 Gy IFR (early stage favorable [ES-F] G4 protocol). Patients with Ann Arbor early stage (I/II) disease and unfavorable clinical risk factors—bulky disease $\geq$ 10 cm or mediastinal mass ratio of $\geq$ 0.33x and/or B symptoms—were treated with 12 weeks Stanford V for 12 weeks and 36 Gy IFR to sites $>$ 5 cm (early stage unfavorable [ES-U] G2 protocol). Patients with advanced stage (AS) III/IV disease were also treated with 12 weeks Stanford V and 36 Gy IFR to sites $>$ 5 cm and the spleen, if involved (AS G3 protocol). One asymptomatic patient with early-stage nonbulky disease was treated on the G2 protocol because of an elevated erythrocyte sedimentation rate and involvement of more than three nodal sites. Three additional patients with early-stage bulky disease on physical examination were treated on the G4 protocol. Long-term follow-up (median, 9 years) and detailed clinical information were available on all patients.

FISH

FISH was performed as previously described.[13,18] In brief, bacterial artificial chromosome (BAC) clones were selected from the UCSC Genome Browser and ordered from BACPAC Resources Center at Children's Hospital Oakland Research Institute in Oakland, California (https://bacpac.chori.org/home.htm). BAC DNA was extracted from Luria broth cultures by using the Qiagen Large-Construct Kit (Hilden, Germany) according to the manufacturer's recommendations and nick labeled with standard protocols (Abbott Molecular, Des Plaines, IL). Probes included Spectrum Orange–labeled RP11-599H20, which maps to 9p24.1 and includes *CD274*/*PD-L1*; Spectrum Green–labeled RP11-635N21, which also maps to 9p24.1 and encompasses *PDCD1LG2*/*PD-L2*; and Spectrum Aqua–labeled CEP9, a control centromeric probe that maps to 9p11-q11 (Abbott Molecular). An additional probe, Spectrum Green–labeled RP11-610G2, which maps upstream of *PDCD1LG2*, was used to confirm a possible chromosomal translocation.

Hematoxylin and eosin–stained FFPE tissue sections were reviewed, RS cells identified by their nuclear morphologic features, and areas with the highest density of RS cells circled by an expert hematopathologist (S.J.R.). Thereafter, slides were hybridized according to the manufacturer's recommendations (Abbott Molecular). Approximately 50 RS cells per case were analyzed. Nuclei with a target:control probe ratio of $\geq$ 3:1 were defined as amplified, and those with a probe ratio of $>$ 1:1 but $<$ 3:1 were classified as relative copy gain. In certain instances, cells with aggregated target signals that were tightly clustered around the control signal were classified as amplified by an expert cytogeneticist (A.H.L.). Nuclei with a probe ratio of 1:1 but more than two copies of each probe were defined as polysomic for chromosome 9p. In each case, the percent and magnitude of 9p24.1 amplification, copy gain, polysomy, and normal copy numbers (disomy) were noted. Cases were classified by the highest observed level of 9p24.1 alteration. Specifically, cases with 9p24.1 copy gain lacked amplification, and cases with 9p polysomy lacked 9p24.1 copy gain or amplification.

Double Immunohistochemistry Staining

Double staining of PD-L1 (clone 405.9A11; G.J.F.[19]) and PAX5 (24/Pax-5; BD Biosciences, San Jose, CA) and of PD-L2 (clone 366C.9E5; G.J.F.) and pSTAT3 (D3A7; Cell Signaling, Danvers, MA) was performed with an automated staining system (Bond III; Leica Biosystems, Buffalo Grove, IL) as previously described.[13] Stained slides were scored by an expert hematopathologist (S.J.R.), and average intensity of staining (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) was reported. PD-L1 expression in PAX5 dim-positive malignant RS cells and PAX5-negative infiltrating normal cells was assessed separately. For PD-L1, 50 RS cells were counted, the number of malignant cells with positive staining (average intensity, 1 to 3+) was determined, and the percentage of positive cells was calculated (0% to 100%). For PD-L2 and pSTAT3, the percentage of malignant and nonmalignant cells with positive staining for PD-L2 within the tissue section was estimated (0% to 100%), and the average intensity of staining scored (1 to 3+). A modified H-score was generated by multiplying the percentage of malignant cells with positive staining (0% to 100%) and average intensity of positive staining in RS cells (1 to 3+).

EBV-Encoded Small RNA In Situ Hybridization

EBV-encoded small RNA (EBER) in situ hybridization was performed with an automated staining system (Bond III; Leica Biosystems) while following the manufacturer's protocol. Briefly, 4-μm-thick paraffin-embedded sections were prebaked at 60°C for 1 hour. Slides were loaded on the Bond III, dewaxed, rehydrated, and pretreated with a diluted enzyme solution for 15 minutes (Enzyme 1; Leica Biosystems). Thereafter, slides were incubated with a fluorescein-conjugated oligonucleotide EBER probe

(600 ng/mL; Leica Biosystems) at 42°C for 2 hours. Subsequently, an antifluorescein antibody (Leica Biosystems) was applied to the slides for 15 minutes, followed by 8 minutes of postprimary blocking reagent, 8 minutes of horseradish peroxidase–labeled polymer, and 5 minutes of peroxidase block. Slides were then developed with 3,3-diaminobenzidine (10 minutes), counterstained with hematoxylin (5 minutes), dehydrated, and coverslipped. The aforementioned reagents were all components of the Bond Polymer Refine Detection system (Leica Biosystems).

Statistical Analysis

Analyses of 9p24.1 alterations, PD-L1 and PD-L2 protein expression, and EBER status were performed while blinded to the clinical data. Clinical characteristics of the patients with cHL were assessed by using descriptive statistics. Associations between variables were evaluated with Fisher exact test for categorical data and Wilcoxon or Kruskal-Wallis rank sum test for continuous data that compared two or more groups, respectively. The modified H-score for PD-L1 and PD-L2 protein expression was divided into four equally sized groups (quartiles), and locally weighted polynomial smoothing was used to fit a trend line over the data. All *P* values were two-sided. Progression-free survival (PFS) was defined from the date of diagnosis until the date of relapse or death in the absence of relapse or was censored at the date of last contact. Time-to-event analyses were performed by using the Kaplan-Meier method, and errors were calculated by Greenwood formula. Differences in survival curves were assessed with log-rank tests. Multivariable Cox proportional hazards models were fit and evaluated by using likelihood ratio tests. Two-sided $P < .05$ was considered statistically significant, and no corrections for multiple comparisons were performed.

RESULTS

Patient Characteristics

The characteristics of the 108 patients with newly diagnosed cHL are summarized in Table 1. The median age was 30 years, and the majority of patients (93 of 108 [86%]) had nodular sclerosis HL, 11% (12 of 108) had mixed-cellularity HL, and 3% (three of 108) had cHL not otherwise specified. Patients were classified on the basis of disease stage and the presence or absence of B symptoms and/or bulky disease as ES-F (no bulky disease or B symptoms, n = 33), ES-U (bulky disease and/or B symptoms, n = 41), or AS (n = 34).

Genetic Analyses of the PD-L1 and PD-L2 Loci

A recently developed FISH assay was used to characterize 9p24.1/*PD-L1*/*PD-L2* alterations in diagnostic FFPE tumor specimens from each patient (Fig 1A).[13,18] RS cells were scored as having 9p24.1 disomy, polysomy, copy gain, or amplification, and the magnitude of 9p24.1 gain and percentage of cells with each alteration was noted (representative images in Fig 1B). Cases were classified by the highest observed level of 9p24.1 alteration.

Frequency of the 9p24.1 Alterations in cHL

Almost all of the 108 patients in this series had concordant alterations of the *PD-L1* and *PD-L2* loci in their diagnostic biopsy specimens. Only one patient (1%) had normal 9p24.1 copy numbers (disomy), and five (5%) had polysomy of 9p. In marked contrast, 56% (61 patients) had 9p24.1 copy gain, and 36% (39 patients) had 9p24.1 amplification (Table 2). There was an association between PD-L1 and PD-L2 protein expression and 9p24.1 genetic alterations in the RS cells (Fig 1C). RS cells also expressed pSTAT3, indicative of active JAK-STAT signaling (Fig 1C). Of note, two of the cHL cases had a chromosomal rearrangement of 9p24.1 detected by a split of the red and green FISH signals (Table 2; Appendix Fig A1, online only).

Spectrum of 9p24.1 Alterations in cHL

By analyzing each case with FISH, we were able to assess the full spectrum of 9p24.1 alterations in each tumor. In cases classified by the highest observed level of 9p24.1 alteration, we also identified RS cells with lower-level 9p24.1 CNAs (Table 2; Appendix Table A1; Fig 2A). Specifically, all cases classified as having 9p24.1 amplification had additional RS cells with 9p24.1 copy gain (2% to 82% of cells), 9p polysomy (2% to 52% of cells), and/or 9p24.1 residual disomy (2% to 35% of cells;

Table 1. Clinical Characteristics and Pathologic Subtypes

Characteristic	All (N = 108)	Early Stage Favorable (n = 33)	Early Stage Unfavorable (n = 41)	Advanced Stage (n = 34)
Age, years				
Median (range)	30 (18-69)	30 (18-56)	29 (18-66)	29 (19-69)
Stage, No. (%)				
I	9 (8)	8 (24)	1 (2)	—
II	65 (60)	25 (76)	40 (98)	—
III	22 (20)	—	—	22 (65)
IV	12 (11)	—	—	12 (35)
Bulky, No. (%)				
> 10 cm	16 (15)	—	12 (29)	4 (12)
≥ 0.33x mediastinal mass ratio	19 (18)	—	11 (27)	8 (24)
Both	15 (14)	—	8 (20)	7 (21)
B symptoms, No. (%)	39 (36)	—	18 (44)	21 (62)
Histologic subtype, No. (%)				
Nodular sclerosis	93 (86)	25 (76)	39 (95)	29 (85)
Mixed cellularity	12 (11)	8 (24)	1 (2)	3 (9)
cHL, not otherwise specified	3 (3)	—	1 (2)	2 (6)

Abbreviation: cHL, classical Hodgkin lymphoma.

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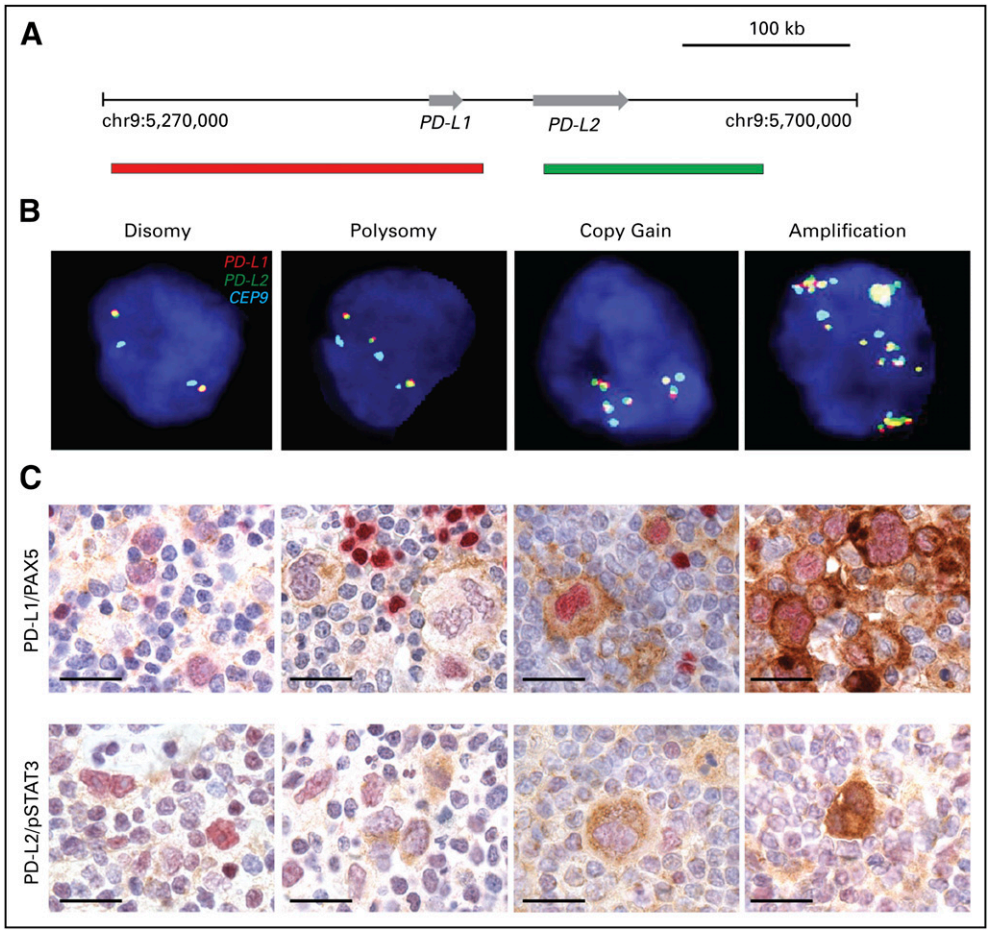

Fig 1. Genetic and immunohistochemical analyses of the *PD-L1* and *PD-L2* loci and PD-1 ligand expression. (A) Location and color labeling of the bacterial artificial chromosome (chr) clones on 9p24.1 used for fluorescent in situ hybridization (FISH). RP11-599H20 including PD-L1, labeled red. RP11-635N21 including PD-L2, labeled green. (B) Representative images of FISH results for the various categories. PD-L1 in red, PD-L2 in green, fused (F) signals in yellow, and centromeric probe (CEP9) in aqua (A). In these images, disomy reflects 2A:2F; polysomy, 3A:3F; copy gain, 3A:6F; and amplification, 15+F. (C) The top panel shows PD-L1 (brown)/PAX5 (red) immunohistochemistry (IHC) in the classical Hodgkin lymphoma (cHL) cases with 9p24.1 disomy, polysomy, copy gain, and amplification from (B). The bottom panel shows PD-L2 (brown)/pSTAT3 (red) IHC in the same cHL cases. Scale bar = 50 μm.

Table 2; Appendix Table A1; Fig 2A). Similarly, cases identified as having 9p24.1 copy gain included additional RS cells with 9p polysomy (4% to 78% of cells) and/or 9p24.1 residual disomy (2% to 86% of cells; Table 2; Appendix Table A1; Fig 2A). In cases classified as polysomic for chromosome 9p, additional RS cells were disomic for 9p24.1 (66% to 93% of cells; Table 2; Appendix Table A1; Fig 2A).

As shown in Figure 2A, there was a spectrum of 9p24.1 alterations in the evaluated cHL series that ranged from low-level polysomy (6% polysomic RS cells) to near-uniform 9p24.1 amplification (92% amplified RS cells). Consistent with the ordered spectrum of 9p24.1 alterations in this series (Fig 2A), the percentage of residual 9p24.1 disomic cells was highest in cases classified as polysomic for 9p, intermediate in tumors with 9p24.1 copy gain, and lowest in tumors with 9p24.1 amplification ($P < .001$, Kruskal-Wallis test; Fig 2B).

9p24.1 Alterations and PD-1 Ligand Expression

After characterizing the spectrum of 9p24.1 CNAs in the cHL series, we assessed the relationship between these alterations and expression of the PD-1 ligands. Given the inverse relationship between 9p24.1 alterations and residual 9p24.1 disomy (Figs 2A

Table 2. *PD-L1* and *PD-L2* Genetic Alterations

				Additional Alterations							
				Disomy		Polysomy		Copy Gain		Amplification	
Cytogenetics	Patients (N = 108), No. (%)	% Positive Cells per Case	Median No. of Copies	No. of Cases/ Total (%)	% Cells	No. of Cases/ Total (%)	% Cells	No. of Cases/ Total (%)	% Cells	No. of Cases/ Total (%)	% Cells
Disomy	1 (1)	100	2	NA	NA	—	—	—	—	—	—
Polysomy	5 (5)	6-34	3	5/5 (100)	66-94	NA	NA	—	—	—	—
Copy gain	61 (56)	4-100	6	58/61 (95)	2-86	57/61 (93)	4-78	NA	NA	—	—
Amplification	39 (36)	2-92	10+	35/39 (90)	2-35	25/39 (64)	2-52	39/39 (100)	2-82	NA	NA
Translocation	2 (2)	72-88	NA	2/2 (100)	4-22	2/2 (100)	6-8	—	—	—	—

Abbreviation: NA, not applicable.

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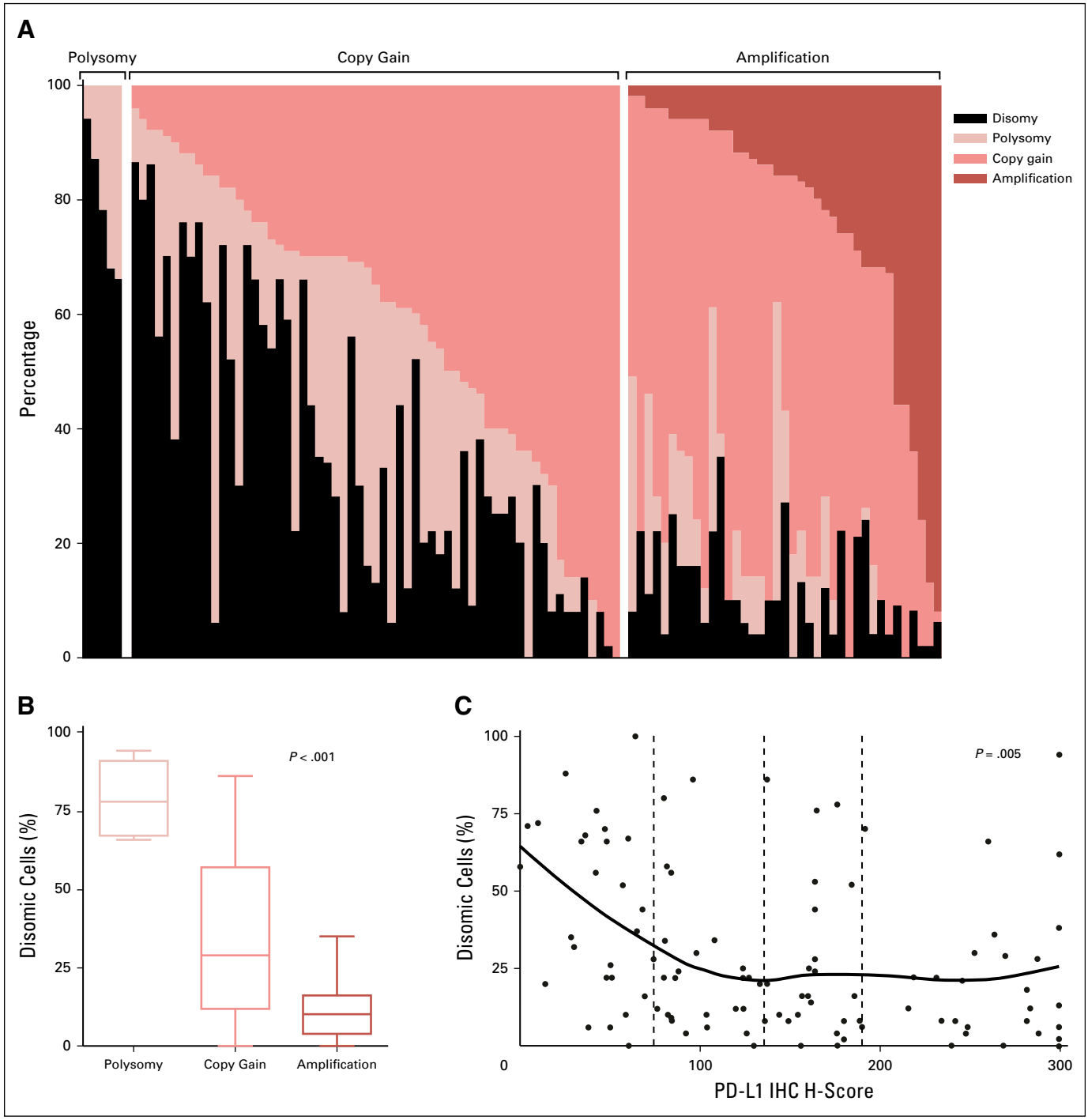

Fig 2. The spectrum of 9p24.1 alterations in classical Hodgkin lymphoma (cHL). (A) 9p24.1 alterations in evaluated cHLs. The cHLs are classified by the highest observed level of 9p24.1 alteration in Reed-Sternberg (RS) cells: polysomy, copy gain, or amplification (top). Individual tumors are depicted as columns on the *x*-axis. In each cHL, the percentage of RS cells with 9p24.1 disomy (black), polysomy (light pink), copy gain (pink), and/or amplification (red) is shown on the *y*-axis. (B) Percentage of RS cells with residual 9p24.1 disomy in cHLs classified by 9p24.1 alterations, as represented as box-and-whisker plots, showing minimum, first quartile, median, third quartile, and maximum. cHLs with 9p24.1 polysomy, copy gain, and amplification have significantly different percentages of residual RS cells with normal (disomic) 9p24.1 copy numbers. $P < .001$, Kruskal-Wallis test. (C) Association of PD-L1 protein expression and 9p24.1 copy number alterations. Residual 9p24.1 disomy is depicted on the *y*-axis; PD-L1 immunohistochemistry (IHC) H-score (in quartiles) is shown on the *x*-axis. Quartiles are indicated by dashed lines. A locally weighted polynomial regression line is shown in black. A highly significant decrease in percentage of residual 9p24.1 disomic cells in cHLs with a higher PD-L1 IHC H-score is shown. $P = .005$, Kruskal-Wallis test.

and 2B), we used residual 9p24.1 disomy and the PD-L1 H-score (percentage of malignant cells with positive staining multiplied by the average intensity of positive staining, divided into quartiles) for these analyses. A highly significant association was found between decreased residual 9p24.1 disomy and increased PD-L1 expression ($P = .005$, Kruskal-Wallis test; Fig 2C; Appendix Fig A2A). Similar

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results were obtained for PD-L2 protein expression (Appendix Figs A2B and A2C).

The distribution of genetic alterations in patients with EBV-negative and EBV-positive cHL was similar in this series (Appendix Fig A3). However, EBV-positive cHLs were more likely to have high PD-L1 H-scores (Appendix Fig A3), indicating further induction of PD-L1 expression by viral infection.

9p24.1 Alterations, Clinical Risk Factors, and Outcome

After characterizing the *PD-L1/PD-L2* alterations in this series of patients, we assessed potential associations among these genetic lesions, clinical risk factors, and outcome. First, PFS was assessed for patients with ES-F, ES-U, and AS disease. The outcome for patients with ES-F and ES-U disease was comparable possibly partly due to the more-aggressive treatment of ES-U disease (Fig 3A). As expected, patients with AS disease had a significantly inferior outcome compared with those with ES disease ($P = .002$, log-rank test; Fig 3A). We next assessed the PFS for patients by 9p24.1 genetic alteration and identified significant differences in outcome that were most striking for patients with 9p24.1 amplification ($P < .001$, log-rank test; Fig 3B).

The significantly decreased PFS in patients with AS disease (Fig 3A) and 9p24.1 amplification (Fig 3B) prompted us to assess the distribution of 9p24.1 alterations in the clinical risk groups (Figs 3C [all patients] and 3D [risk groups]). The incidence of 9p24.1 amplification increased by clinical risk group (ES-F, 24%; ES-U, 34%; AS, 50%; $P = .024$, Kruskal-Wallis test; Fig 3D). We next determined the effect of 9p24.1 amplification on PFS in the various clinical risk groups. Although the small numbers limited the statistical analysis, there was a trend toward worse outcome in patients with ES-U and AS disease who had 9p24.1 amplification (Appendix Fig A4).

We next assessed the independent prognostic significance of the clinical risk factors, ES-U and AS, and 9p24.1 amplification in Cox univariable and multivariable models. In respective univariable models, the clinical risk factor, AS disease, and 9p24.1 amplification had independent prognostic significance ($P = .017$ and .02, respectively; Table 3 [top and middle panels]). Despite the association of 9p24.1 amplification with AS disease (Fig 3D), the

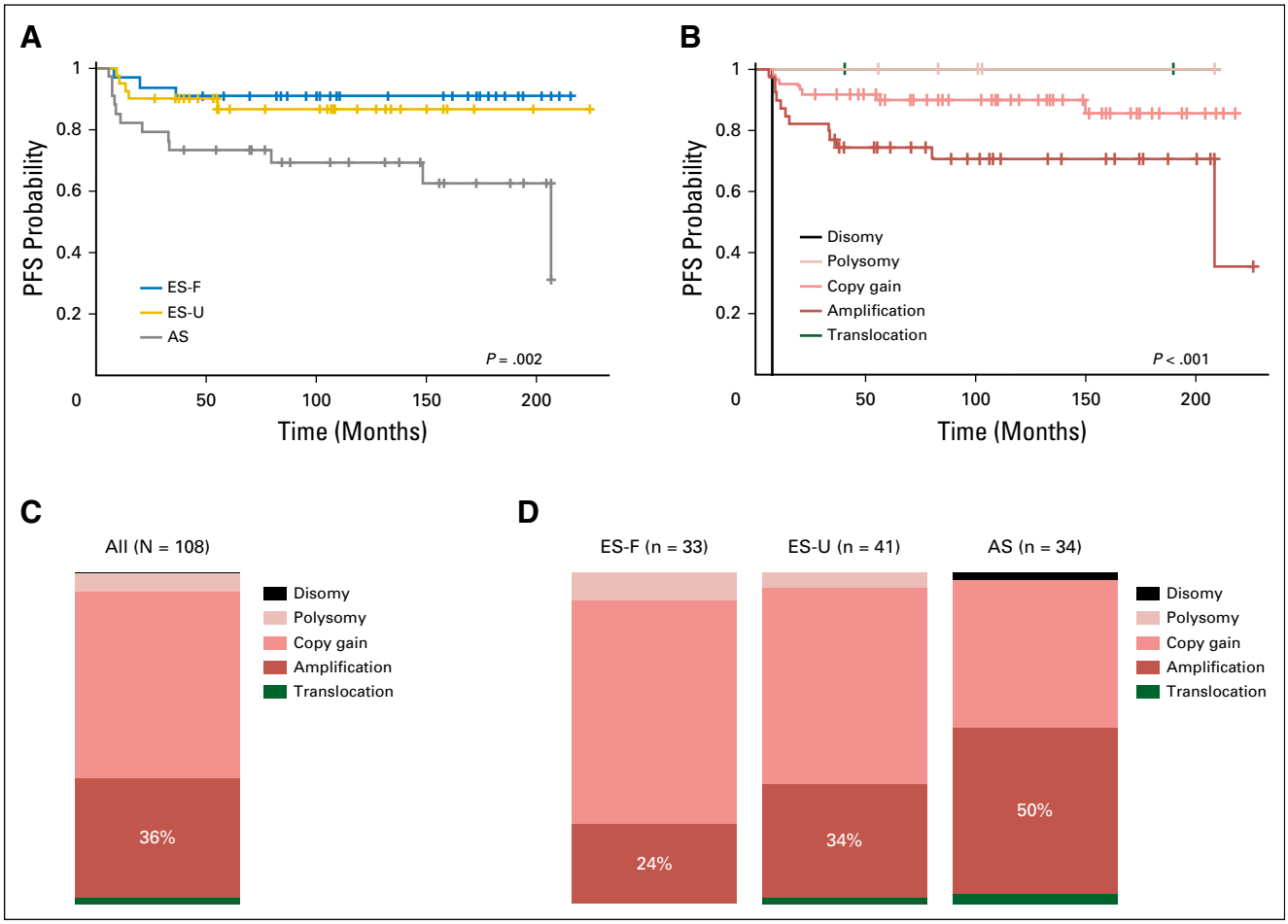

Fig 3. Clinical and genetic predictors of progression-free survival (PFS). (A) PFS by clinical stage in patients with classical Hodgkin lymphoma (cHL), early stage favorable (ES-F; n = 33), early stage unfavorable (ES-U; n = 41), and advanced stage (AS; n = 34). $P = .002$, log-rank test. (B) PFS by 9p24.1 alterations in patients with cHL (disomy, n = 1; polysomy, n = 5; copy gain, n = 61; amplification, n = 39; translocation, n = 2; $P < .001$, log-rank test). (C) Percentage of patients with 9p24.1 disomy (1%), polysomy (5%), copy gain (56%), amplification (36%), and translocation (1%) in the current series. (D) Frequency of 9p24.1 alterations (polysomy, copy gain, amplification, translocation, or disomy) by clinical stage (ES-F, ES-U, and AS) in this series. The incidence of 9p24.1 amplification is significantly different in clinically staged patients (ES-F, 24%; ES-U, 34%; AS, 50%; $P = .024$, Kruskal-Wallis test).

Table 3. Clinical and Genetic Univariable and Multivariable Risk Models

Variable	HR	Lower 95% CI	Upper 95% CI	*P*
Clinical factors*				
Stage I/II, B symptoms and/or bulky disease	1.59	0.377	6.703	.53
Stage III/IV	4.68	1.319	16.616	.017
Presence of amplification†				
Yes	2.9	1.186	7.106	.02
Full model‡				
Stage I/II, B symptoms and/or bulky disease	1.43	0.339	6.041	.63
Stage III/IV	3.76	1.035	13.662	.044
Amplification	2.292	0.919	5.721	.075

Abbreviation: HR, hazard ratio.
*The clinical stages (early stage favorable, early stage unfavorable, advanced stage [AS]) were used to classify patients into risk groups for progression-free survival. The HR estimate for patients with AS (III/IV) disease was significant (P = .017).
†The presence or absence of 9p24.1 amplification was used to classify patients into risk groups for progression-free survival. In a univariable Cox model, 9p24.1 amplification was significant (P = .02).
‡The multivariable Cox model includes the risk factors with independent prognostic significance (9p24.1 amplification and AS disease) and the additional clinical risk factors that influenced treatment (stage I/II, B symptoms, and/or bulky disease). In this multivariable model, the HR for AS retains significance (P = .044; P for amplification = .075).

genetic alteration further delineated PFS in a multivariable model (P = .075; Table 3).

DISCUSSION

We use a 9p24.1 FISH assay to determine the incidence, nature, and prognostic significance of *PD-L1* and *PD-L2* alterations in a series of patients with cHL who were uniformly treated and have long-term outcome data. Almost all the cases in this series had concordant alterations of the *PD-L1* and *PD-L2* loci, which included copy gain and amplification in the majority of tumors (56% and 36%, respectively). There was a spectrum of 9p24.1 alterations in the analyzed cases that ranged from low-level polysomy to near-uniform 9p24.1 amplification. Of note, PFS was significantly shorter for patients with 9p24.1 amplification who were also more likely to have AS disease.

The current studies indicate that *PD-L1*/*PD-L2* CNAs are a defining feature of cHL, seen with near uniformity in patients evaluated with the FISH assay. Previous genetic analyses of cHL were hampered by the rarity of RS cells in primary tumors. As a consequence, analyses of genetic alterations in cHL required laser capture microdissection of malignant RS cells.[6,20,21] In these studies, which relied on array-based comparative genomic hybridization or quantitative polymerase chain reaction, the frequency of *PD-L1*/*PD-L2* CNAs was approximately 40% to 50%.[6,20,21] In such analyses, laser capture microdissected RS specimens included surrounding residual normal tissue, which likely caused an underestimate in *PD-L1*/*PD-L2* copy numbers.

The current studies also indicate that 9p24.1 alterations are subclonal in a subset of primary cHLs. Specifically, these tumors exhibited the full spectrum of 9p24.1 alterations, which ranged from low-level polysomy (6% polysomic RS cells) to near-uniform 9p24.1 amplification (92% amplified RS cells). Consistent with these findings, the percentage of residual 9p24.1 disomic cells was inversely related to *PD-L1*/*PD-L2* CNAs and highest in cHLs classified as polysomic for 9p, intermediate in tumors with 9p24.1 copy gain, and lowest in tumors with 9p24.1 amplification. In cHLs with significant residual disomy, platform-based approaches, such as assay-based comparative genomic hybridization or high-density single nucleotide polymorphism array, or quantitative polymerase chain reaction analyses are likely to underestimate the frequency of 9p24.1 alterations.[6,20,21] These findings may also explain the lower incidence of 9p24.1 CNAs in a small series of flow-sorted $CD30^+$ RS cells evaluated by whole-exome sequencing.[22]

Although 9p24.1 alterations were identified in almost all patients with cHL in this series, the highest-level lesion—amplification—was more common in patients with AS disease. This finding suggests that PD-1–mediated immune evasion may limit local containment and foster tumor spread. In addition, the data provide the rationale for evaluating PD-1 blockade in the frontline setting in patients with AS cHL who may have less-favorable outcomes with standard empirical combination chemotherapy. Given these findings, further analyses of the 9p24.1 alteration in patients treated with standard cHL induction regimens or checkpoint blockade are warranted.

The high frequency of 9p24.1 alterations in cHL prompted further assessment of the *PD-L1* and *PD-L2* loci in other lymphoid malignancies. Several lymphomas have been found to have frequent *PD-L1*/*PD-L2* CNAs and additional chromosomal translocations of the same loci. Like cHL, certain large B-cell lymphoma subtypes, including primary mediastinal large B-cell lymphoma, primary CNS lymphoma, and primary testicular lymphoma, often have *PD-L1*/*PD-L2* CNAs or chromosomal rearrangements and increased expression of the PD-1 ligands.[6,18,23,24] In marked contrast, systemic diffuse large B-cell lymphomas rarely exhibit 9p24.1/*PD-L1*/*PD-L2* CNAs and infrequently express the associated PD-1 ligands.[18,25,26]

Taken together, these data support a strategy for identifying lymphoid malignances with genetic bases for PD-1–mediated tumor immune evasion. In cHL, the near-uniform alterations of the *PD-L1*/*PD-L2* loci likely explain the remarkable activity of PD-1 blockade in this disease.[13]

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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GLOSSARY TERMS

copy number alteration (CNA): a structural variation in the genome with an increased (amplification) or decreased (deletion) number of copies of a gene or region.

Epstein-Barr virus (EBV): virus belonging to the herpes family of viruses. EBV is also called human herpes virus 4 and is an oncogenic virus that is responsible for B-cell transformation. It is associated with Hodgkin lymphoma, immunoblastic B-cell lymphomas, Burkitt's lymphoma, and nasopharyngeal carcinoma.

FISH (fluorescent in situ hybridization): in situ hybridization is a sensitive method generally used to detect specific gene sequences in tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. FISH uses a fluorescent probe to increase the sensitivity of in situ hybridization.

PD-1: programmed cell death protein 1 (CD279), a receptor expressed on the surface of activated T, B, and NK cells that negatively regulates immune responses, including autoimmune and antitumor responses.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

_PD-L1_ and _PD-L2_ Genetic Alterations Define Classical Hodgkin Lymphoma and Predict Outcome

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Travel, Accommodations, Expenses: Bristol-Myers Squibb

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Appendix

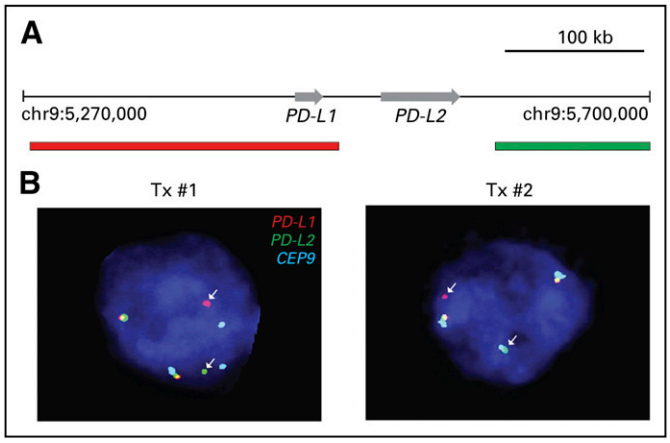

Fig A1. Chromosomal rearrangements in classical Hodgkin lymphoma (cHL). (A) Location and color labeling of the bacterial artificial chromosome (chr) clones on 9p24.1 used for fluorescent in situ hybridization (FISH) in translocation 2 (Tx #2). RP11-599H20 including *PD-L1*, labeled red; RP11-610G2 downstream of *PD-L2*, labeled green. For labeling of bacterial artificial chromosome clones used for translocation 1 (Tx #1), see Figure 1A. (B) FISH analyses of the cHL cases with chromosomal rearrangements. *PD-L1* in red, *PD-L2* in green, and centromeric probe (CEP9) in aqua. Arrows indicate the rearranged allele.

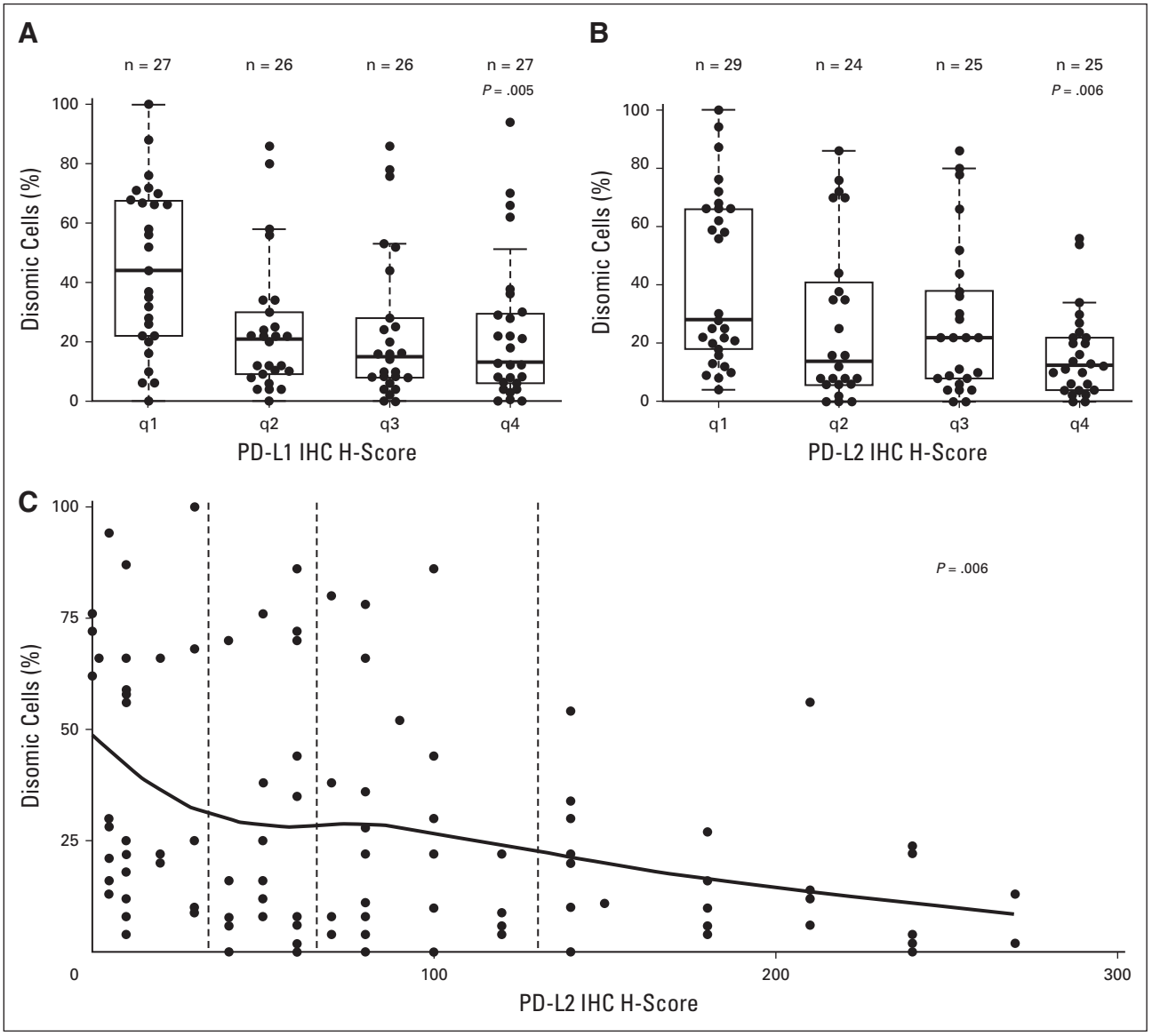

Fig A2. Association of PD-L1/PD-L2 protein expression and 9p24.1 copy number alterations. (A) Percentage of 9p24.1 disomic cells in each of the PD-L1 immunohistochemistry (IHC) H-score quartiles. The *y*-axis shows the percentage of residual 9p24.1 disomic cells; the *x*-axis shows PD-L1 IHC H-score in quartiles. A statistically significant decrease in the percentage of normal (disomic) cells in the H-score quartiles was found. $P = .005$, Kruskal-Wallis test. (B) Percentage of residual 9p24.1 disomic cells in each of the PD-L2 IHC H-score quartiles. The *y*-axis shows the percentage of residual 9p24.1 disomic cells; the *x*-axis shows the PD-L2 IHC H-score in quartiles. The percentage of residual 9p24.1 disomic cells is statistically different in the quartiles. $P = .006$, Kruskal-Wallis test. (C) Percentage of residual 9p24.1 disomic cells (*y*-axis) and PD-L2 IHC H-score (*x*-axis) plotted for individual cases. Quartiles are indicated with dashed lines, and a trend line (locally weighted polynomial regression line) is shown in black. q, quartile.

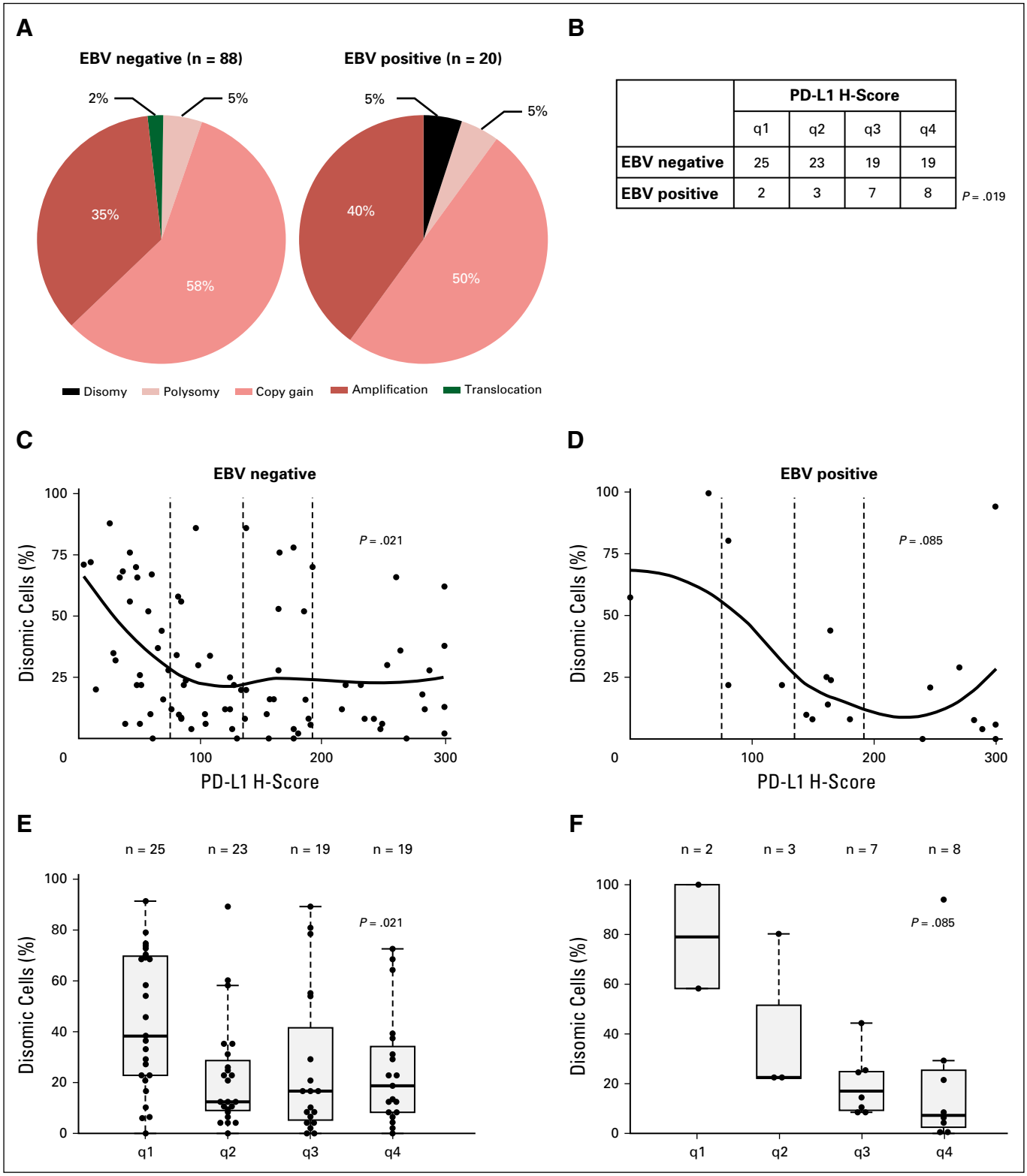

Fig A3. Distribution of genetic alterations in patients with Epstein-Barr virus (EBV) –negative and EBV-positive classical Hodgkin lymphoma (cHL). (A) The status of *PD-L1* and *PD-L2*—disomy, polysomy, copy gain, amplification, and translocation—in EBV-negative (n = 88) and EBV-positive (n = 20) cHLs is visualized with a pie chart. (B) Distribution of EBV-negative and EBV-positive cases in the various PD-L1 immunohistochemistry (IHC) H-score quartiles. The proportion of EBV-positive cases increases as the H-score quartile category increases. $P = .019$, Kruskal-Wallis test. (C) and (D) Percentage of 9p24.1 residual disomic cells (*y*-axis) and PD-L1 IHC H-score (*x*-axis) plotted for individual EBV-negative and EBV-positive cHLs, respectively. Quartiles are indicated with dashed lines, and a trend line (locally weighted polynomial regression line) is shown in black. (E) and (F) Percentages of residual 9p24.1 disomic cells in EBV-negative and EBV-positive cases in the respective PD-L1 IHC H-score quartiles from (C) and (D) are plotted. The percentage of residual 9p24.1 disomic cells is significantly different in the respective H-score quartiles in EBV-negative cHLs in (E). $P = .021$, Kruskal-Wallis test. q, quartile.

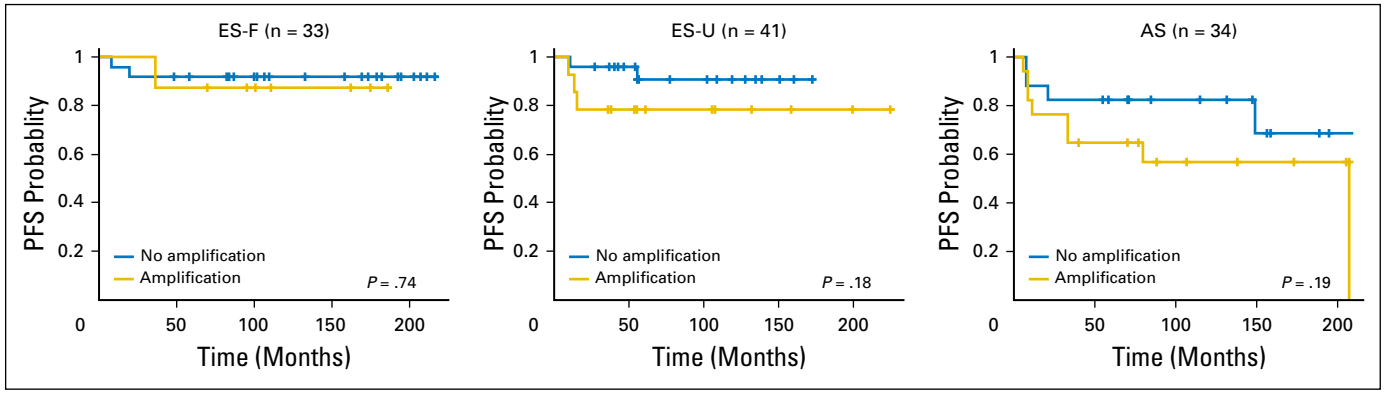

Fig A4. Progression-free survival (PFS) curves for the clinical risk groups with (gold) or without (blue) 9p24.1 amplification: early stage favorable (ES-F; n = 8 and n = 25, respectively), early stage unfavorable (ES-U; n = 14 and n = 27, respectively), and advanced stage (AS; n = 17 and n = 17, respectively).

Table A1. 9p24.1 Fluorescent In Situ Hybridization Analyses in the 108 Patients With Classical Hodgkin Lymphoma

Study ID	No. of RS Cells	Disomy (%)	Polysomy (%)	No. of Copies	Copy Gain (%)	No. of Copies	Amplification (%)	No. of Copies	Translocation (%)	No. of Copies
1	40	87	13	3	—	—	—	—	—	—
2	46	78	22	3	—	—	—	—	—	—
3	50	68	32	3-5	—	—	—	—	—	—
4	50	94	6	3	—	—	—	—	—	—
5	50	66	34	3-4	—	—	—	—	—	—
6	51	86	10	3	4	3-4F:2-3A	—	—	—	—
7	50	80	14	3	6	3F:2A	—	—	—	—
8	50	56	36	3-5	8	3-5F:2-4A	—	—	—	—
9	50	76	12	3	12	3-4F:2-3A	—	—	—	—
10	50	72	6	3-4	22	3-6F:2-5A	—	—	—	—
11	49	59	12	3-5	29	3-5F:2-4A	—	—	—	—
12	49	22	49	3-5	29	3-5F:2-4A	—	—	—	—
13	51	30	39	3-6	31	2-5F:1-4A	—	—	—	—
14	48	13	52	3-6	35	3-8F:2-5A	—	—	—	—
15	49	33	29	3-5	38	3-7F:2-4A	—	—	—	—
16	41	44	17	3	39	3-4F:2-3A	—	—	—	—
17	50	20	38	3-6	42	3-10+F:2-8A	—	—	—	—
18	50	22	28	3-7	50	3-8F:1-6A	—	—	—	—
19	47	9	38	3-6	53	1-9F:1-6A	—	—	—	—
20	54	28	11	3-4	61	3-6F:1-4A	—	—	—	—
21	50	20	12	3	68	3-5F:1-3A	—	—	—	—
22	50	8	22	3-4	70	3-10+F:2-7A	—	—	—	—
23	52	11	6	3	83	3-7F:1-4A	—	—	—	—
24	50	8	6	3	86	3-6F:2-4A	—	—	—	—
25	50	8	6	3	86	3-5F:2-4A	—	—	—	—
26	50	2	—	—	98	3-10+F:2-8A	—	—	—	—
27	51	—	—	—	100	4-10+F:2-8A	—	—	—	—
28	50	10	12	3-4	66	3-9F:1-4A	12	7-9F	—	—
29	50	6	8	3-4	74	3-9F:2-7A	12	10+-15+F	—	—
30	48	4	10	4-6	73	1-10F:1-8A	13	5-10+F	—	—
31	49	27	16	3-4	41	3-5F:1-4A	16	3-7F	—	—
32	50	—	—	—	74	3-8F:2-6A	26	6-10+F	—	—
33	50	24	2	3	42	3-8F:2-5A	32	3-10+F	—	—
34	50	10	—	—	58	3-9F:1-4A	32	3-10+F:1-6A	—	—
35	50	—	—	—	44	3-7F:2-3A	56	6-10+F	—	—
36	50	86	6	3	8	3-4F:2A	—	—	—	—
37	44	70	21	3-4	9	3-4F:2A	—	—	—	—
38	50	70	18	3-4	12	3F:2A	—	—	—	—
39	50	76	10	3-4	14	3-6F:2-3A	—	—	—	—
40	50	6	78	3-7	16	3-7F:2-6A	—	—	—	—
41	50	72	10	3	18	3-4F:1-3A	—	—	—	—
42	33	52	30	3-4	18	3-5F:2-4A	—	—	—	—
43	54	30	50	3-5	20	3-9F:2-6A	—	—	—	—
44	38	58	18	3	24	3-4F:2-3A	—	—	—	—
45	37	54	19	3	27	3-5F:2-4A	—	—	—	—
46	50	66	4	3	30	3-4F:1-2A	—	—	—	—
47	50	44	26	3-4	30	3-6F:2-3A	—	—	—	—
48	50	34	36	3-5	30	3-8F:3-5A	—	—	—	—
49	50	28	42	3-5	30	3-6F:2-5A	—	—	—	—
50	50	16	52	3-6	32	3-7F:2-5A	—	—	—	—
51	50	6	56	3-10	38	4-9F:2-7A	—	—	—	—
52	49	12	49	3-6	39	3-8F:2-6A	—	—	—	—
53	50	52	8	3-4	40	3-7F:2-4A	—	—	—	—
54	50	18	36	3-5	46	3-6F:2-4A	—	—	—	—
55	40	25	15	3-5	60	3-5F:1-3A	—	—	—	—
56	55	25	15	3	60	3-6F:2-4A	—	—	—	—
57	50	20	16	3	64	3-6F:1-4A	—	—	—	—
58	50	—	10	3F and 10+F	90	4-15+F:3-10+A	—	—	—	—
59	50	8	—	—	92	3-8F:2-5A	—	—	—	—
60	49	8	41	3-5	49	3-7F:2-5A	2	10+F	—	—
61	51	22	—	—	76	3-6F:2-6A	2	7F	—	—
62	50	22	6	3	68	3-5F:3-4A	4	8F	—	—
63	49	25	14	3	55	3-4F:1-3A	6	5 and 8F:2A	—	—
64	49	16	19	3-6	59	3-10+F:2-7A	6	10+-15+F	—	—
65	51	22	39	3-7	31	3-9F:2-5A	8	3-10+F	—	—
66	51	35	4	3-4	53	3-8F:2-4A	8	3-10+F	—	—
67	50	10	—	—	82	3-5F:2-3A	8	6-10+F	—	—
68	50	10	—	—	76	3-7F:2-4A	14	6-9F	—	—
69	50	12	16	3-5	50	3-15+F:2-6A	22	3-15+F	—	—
70	49	4	6	3	67	3-7+F:2-5A	23	5+F-7+F	—	—
71	50	4	12	3-7	52	3-9F:2-7A	32	6-15+F	—	—
72	43	9	—	—	35	4-9F:2-4A	56	5-10+F	—	—
73	46	2	—	—	11	3-4F:2A	87	5-10+F	—	—

(continued on following page)

Table A1. 9p24.1 Fluorescent In Situ Hybridization Analyses in the 108 Patients With Classical Hodgkin Lymphoma (continued)

Study ID	No. of RS Cells	Disomy (%)	Polysomy (%)	No. of Copies	Copy Gain (%)	No. of Copies	Amplification (%)	No. of Copies	Translocation (%)	No. of Copies
74	52	4	8	3	—	—	—	—	88	One allele rearranged
75	50	100	—	—	—	—	—	—	—	—
76	50	38	52	3-6	10	4-5F:3A	—	—	—	—
77	50	62	22	3-4	16	3-4F:2-3A	—	—	—	—
78	51	66	10	3-4	24	3-5F:1-4A	—	—	—	—
79	50	66	6	3-5	28	3-6F:2-4A	—	—	—	—
80	49	35	35	3-6	30	3-5F:2-4A	—	—	—	—
81	50	8	62	3-8	30	5-7F:3-6A	—	—	—	—
82	48	56	13	3-4	31	3-4F:1-3A	—	—	—	—
83	49	22	33	3-6	45	3-7F:1-5A	—	—	—	—
84	50	12	38	3-4	50	3-7F:2-5A	—	—	—	—
85	50	36	12	3-5	52	3-6F:1-5A	—	—	—	—
86	37	38	8	3-4	54	3-6F:1-4A	—	—	—	—
87	50	28	12	3-5	60	3-5+F:1-4A	—	—	—	—
88	50	—	36	3-5	64	3-9F:2-5A	—	—	—	—
89	50	30	4	3-4	66	3-7F:2-3A	—	—	—	—
90	49	14	—	—	86	3-6F:2-5A	—	—	—	—
91	48	11	35	3-8	50	4-10+F:3-7A	4	4-9F	—	—
92	46	4	16	3-4	76	3-7F:1-5A	4	10+F	—	—
93	50	16	20	3-6	58	3-9F:2-5A	6	6-9F	—	—
94	50	16	8	3-4	70	3-8F:2-4A	6	6F	—	—
95	49	6	6	3	82	3-9F:2-5A	6	9-15+F	—	—
96	49	4	10	3-4	72	3-10+F:2-8A	14	8-10+F	—	—
97	50	10	52	3-10+	22	3-15+F:2-10+A	16	6-15+F	—	—
98	50	—	18	3-7	66	4-8F:2-6A	16	3-10+F	—	—
99	53	13	9	3-10+	61	4-10+F:2-9A	17	5+F-10+F	—	—
100	50	6	8	3	68	3-8F:2-4A	18	6-10+F	—	—
101	50	—	14	3-7	66	1-10+F:2-9A	20	6-15+F	—	—
102	50	22	—	—	52	3-5F:1-2A	26	3-7F	—	—
103	52	21	—	—	50	3-9F:2-4A	29	3-10+F	—	—
104	51	4	—	—	63	3-8F:2-5A	33	6-10+F	—	—
105	50	8	—	—	28	3-5F:1-3A	64	6-15+F	—	—
106	50	2	—	—	22	4-5F:2A	76	6-10+F	—	—
107	50	6	—	—	2	3F:2A	92	3-9F	—	—
108	50	22	6	3	—	—	—	—	72	One allele rearranged

NOTE. The data are color coded by clinical stage: early stage favorable, light blue; early stage unfavorable, medium blue; advanced stage, dark blue.
Abbreviations: A, aqua centromeric signal; F, fused red/green *PD-L1*/*PD-L2* signal; RS, Reed-Sternberg.