9p24.1 alterations and programmed cell death 1 ligand 1 expression in early stage unfavourable classical Hodgkin lymphoma: an analysis from the German Hodgkin Study Group NIVAHL trial

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Summary

High programmed cell death 1 ligand 1 (PD-L1) protein expression and copy number alterations (CNAs) of the corresponding genomic locus 9p24.1 in Hodgkin- and Reed–Sternberg cells (HRSC) have been shown to be associated with favourable response to anti-PD-1 checkpoint inhibition in relapsed/refractory (r/r) classical Hodgkin lymphoma (cHL). In the present study, we investigated baseline 9p24.1 status as well as PD-L1 and major histocompatibility complex (MHC) class I and II protein expression in 82 biopsies from patients with early stage unfavourable cHL treated with anti-PD-1-based first-line treatment in the German Hodgkin Study Group (GHSG) NIVAHL trial (ClinicalTrials.gov Identifier: NCT03004833). All evaluated specimens showed 9p24.1 CNA in HRSC to some extent, but with high intratumoral heterogeneity and an overall smaller range of alterations than reported in advanced-stage or r/r cHL. All but two cases (97%) showed PD-L1 expression by the tumour cells in variable amounts. While MHC-I was rarely expressed in >50% of HRSC, MHC-II expression in >50% of HRSC was found more frequently. No obvious impact of 9p24.1 CNA or PD-L1 and MHC-I/II expression on early response to the highly effective anti-PD-1-based NIVAHL first-line treatment was observed. Further studies evaluating an expanded panel of potential biomarkers are needed to optimally stratify anti-PD-1 first-line cHL treatment.

Keywords: fluorescence *in situ* hybridisation, CD274, classical Hodgkin lymphoma, immune checkpoint blockade, major histocompatibility complex.

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Introduction

The crosstalk between the quantitatively dominant non-neoplastic bystander cells and the tumour cells plays an important role in the pathobiology of classical Hodgkin lymphoma (cHL).[1] On the one hand, the tumour cells receive survival signals from the bystander cells, but on the other hand, the Hodgkin- and Reed–Sternberg cells (HRSC) can apparently prevent an effective anti-tumour immunity by diverse, to date only partially elucidated mechanisms. The latter include altered antigen presentation by diminished expression of major histocompatibility complex class I and II molecules (MHC-I/II) and enhanced programmed death 1 (PD-1) signalling, which are at least partially caused by genetic alterations.[2–5] Based on high efficacy and a favourable safety profile in patients with relapsed or refractory (r/r) cHL,[6–13] several anti-PD-1 antibodies have been approved for the treatment of cHL in this setting. Therapeutic intervention of PD-1 signalling is also an attractive adjunct to increase efficacy and/or to minimise toxicities associated with conventional therapies[14–16] in the first-line setting, and initial studies investigating the potential of an anti-PD-1-based first-line treatment were recently published.[17,18] To optimally harness the therapeutic potential of anti-PD-1 blockade in cHL, it is of immediate interest to robustly identify patients with particular benefit from immune checkpoint inhibition treatment approaches, including predictive biomarkers.[19]

In r/r cHL it has been reported that genomic alterations of the cluster of differentiation 274 [*CD274*; PD-1 ligand 1 (PD-L1)] gene locus on 9p24.1 and the PD-L1 as well as the MHC-II expression of the HRSC determined by immunohistochemistry (IHC) are potential predictors of favourable outcome after PD-1 blockade.[20] In a small study of patients with advanced-stage cHL receiving nivolumab-based first-line treatment (cohort D, CA209-205 trial), more favourable responses to nivolumab monotherapy and deeper and more durable responses to nivolumab and doxorubicin, vinblastine, and dacarbazine (N-AVD) in patients with a higher PD-L1 expression of the HRSC have been reported.[18] An association of 9p24.1 alterations and/or PD-L1 expression with progression-free survival (PFS) after initiation of anti-PD-1 treatment, as observed in the setting of r/r cHL,[20] has not yet been reported in the CA209-205 first-line advanced-stage disease cohort. We recently reported the excellent efficacy of nivolumab and either concomitant or sequential AVD in early stage unfavourable cHL in the German Hodgkin Study Group (GHSG) phase II NIVAHL trial (ClinicalTrials.gov Identifier: NCT03004833).[17,21] In the present study, we investigated 82 tumour specimens of patients with cHL enrolled in this trial for 9p24.1 alterations and protein expression of PD-L1 as well as MHC-I/II in order to determine their potential pathobiological role and their relevance as biomarkers.

Patients and methods

Patient cohort

The tumour samples investigated were obtained from adult patients with treatment-naïve early stage unfavourable cHL treated within the investigator-initiated phase II NIVAHL trial, the detailed primary analysis and protocol of which have been reported recently.[17] Briefly, patients were randomised between either concomitant treatment with four cycles of N-AVD or sequential treatment with four initial doses of nivolumab, two cycles of N-AVD, and two cycles of AVD, followed by 30-Gy involved-site radiotherapy in both groups. A positron emission tomography/computed tomography-based interim staging after two cycles of N-AVD (concomitant group) or four doses of nivolumab monotherapy (sequential group), respectively, was performed to assess early response to (chemo-) immunotherapy.[21] The present analysis includes all patients from the NIVAHL trial with written informed consent for correlative studies and sufficient tissue available, irrespective of clinical characteristics and disease course.

Immunohistochemistry

The IHC was performed according to standard protocols. Details on the used antibodies are given in Table SI. To determine PD-L1 expression, about 50 HRSC were analysed in each case, and PD-L1 expression was categorised into the following groups: >50% of HRSC membranous positive ($HRSC^{+}$), 25–50% of HRSC positive ($HRSC^{+/-}$), <25% of HRSC positive ($HRSC^{-/+}$) and HRSC negative ($HRSC^{-}$). In addition, the expression of MHC-I, using β_2-microglobulin (B2M) as a surrogate,[4,20] and MHC-II, represented by human leucocyte antigen (HLA)-DP/DQ/DR, by IHC was investigated independently by two experienced

haematopathologists (W.K. and A.D.), and discrepancies were resolved in a joint review. For MHC-I/II, we applied the categories previously published[22] and categorised into the following groups: membranous staining in >50% of the tumour cells ($HRSC^+$) and in < 50% of the tumour cells ($HRSC^-$).

Moreover, we have investigated the PD-L1 protein expression of the bystander cells in the proximity of the HRSC and performed digital image analysis of all PD-L1-positive cells (including HRSC and the bystander cells), as described in detail in the Data S1.

Fluorescence in situ hybridization

For the analysis of 9p24.1 copy number alteration (CNA), we used a technique based on a combination of a CD30 IHC staining and a tricolour fluorescence *in situ* hybridisation (FISH) assay. Therefore, serial slides for CD30 IHC and FISH were produced. For evaluation, we used the Bioview System (distributed by Abbott Molecular Inc., Inc. Des Plaines, IL, USA), containing an Olympus Microscope, the Duet™ Scanning Software and the Solo™ Analysing Software. We produced scans of the IHC slide and marked regions of interest (ROIs) containing large amounts of CD30-positive HRSC (Fig 1A). Likewise, a scan of the FISH slide with the 4′,6-diamidino-2-phenylindole filter was recorded. Both of the scanned images (IHC and FISH) were matched at equivalent points and an 'overlay' was produced. Thus, the marks from the CD30 staining were transmitted to the FISH slide image (Fig 1B), and 50 tumour cells per case were analysed in several of the previously selected ROIs (Fig 1C,D). FISH analyses were performed according to standard protocols and described in detail in the Data S1.

The tumour cell nuclei with a PD-L1/2 and enumeration probe ratio of 1:1, but >2 copies of each probe were defined as polysomic; a ratio of >1·0 and <3·0 was defined as copy gain and a ratio ≥3·0 as amplification (in analogy to Ref. [3]). Based on previous reports of 9p24.1 alterations in cHL,[3,20] we assigned the cases for comparison reasons in CNA categories ('gain', 'amplification') by the highest observed level of 9p24.1 alterations in the HRSC, meaning that a case is designated in the 'amplification' category even if only one out of 50 analysed cells fulfil the above defined criteria for an amplification.

In addition, we applied a modified *H*-Score to the FISH data, as described in detail in the Data S1.

Statistical analysis

Data were analysed descriptively. Fisher's exact tests and *t*-tests were used as applicable to explore and quantify differences between subgroups defined by CNA category in terms of clinical and histopathological characteristics and IHC parameters, as well as correlations between CNA and IHC parameters with early response. For regression analyses, IHC parameters were dichotomised as $HRSC^+$ *versus* other. The statistical analysis is described in detail in the Data S1.

Results

Patient characteristics

Of the 109 patients with cHL enrolled in the NIVAHL trial, tumour samples evaluable for FISH analysis were available for 82 patients. The clinical characteristics, histopathological subtypes as well as information on early treatment response of the patients investigated here are summarised in Table I. The group with unspecified cHL subtype was significantly larger in the patients whose samples were not evaluable with FISH (62% vs. 9% in the analysed group), which can be explained by the limited material in these cases, hampering both the subtype assignment and the proper FISH analysis.

9p24.1 CNAs

All patients showed gain of 9p24.1 chromosomal material in HRSC to some extent. In the vast majority of the tumour cells, the counts for the green (PD-L1/upstream) and orange (PD-L2/downstream) probes (9p24.1) were similar in a single cell. However, we observed strong differences in the 9p24.1 genomic status between individual tumour cells in each single case. Within every case, we found among the 50 analysed tumour cells disomic and polysomic cells as well as cells with a variable number of 9p24.1 chromosomal material gains. For comparison reasons, we applied previously reported CNA categories.[3,20] Using these criteria 62 (76%) cases were grouped in the 'copy gain' and 20 (24%) in the 'amplification' category, and Fig 2A depicts the spectrum of 9p24.1 CNA observed. However, among the cases in the 'amplification' category, we found only relatively few numbers of amplified cells in each individual case, ranging from one to eight of the 50 analysed tumour cells that fulfil the determined amplification criteria. The mean [standard deviation (SD)] proportion of disomic cells per patient was 25·4 [15·0]% in the copy gain group and 14·1 [10·4]% in the amplification group respectively (Fig 2B). The *CD274* (PD-L1) gene copy number ranged between 2·5 and 6·9 with means (SDs) of 3·9 (0·8) in the copy gain and 4·9 (1·0) in the amplification group. However, considering the mean ratio of PD-L1 gene copy number and the enumeration probe of all investigated tumour cells per case, no case shows a total value >2. Table II summarises the distribution of the chromosomal alterations in the two CNA categories. We observed no apparent association between these CNA categories and any of the clinical and histopathological characteristics described in Table I.

Potential splits between the green and orange probes as hints for a break between the PD-L1 and PD-L2 locus were not observed in a significant number (>25%) of the tumour cells.

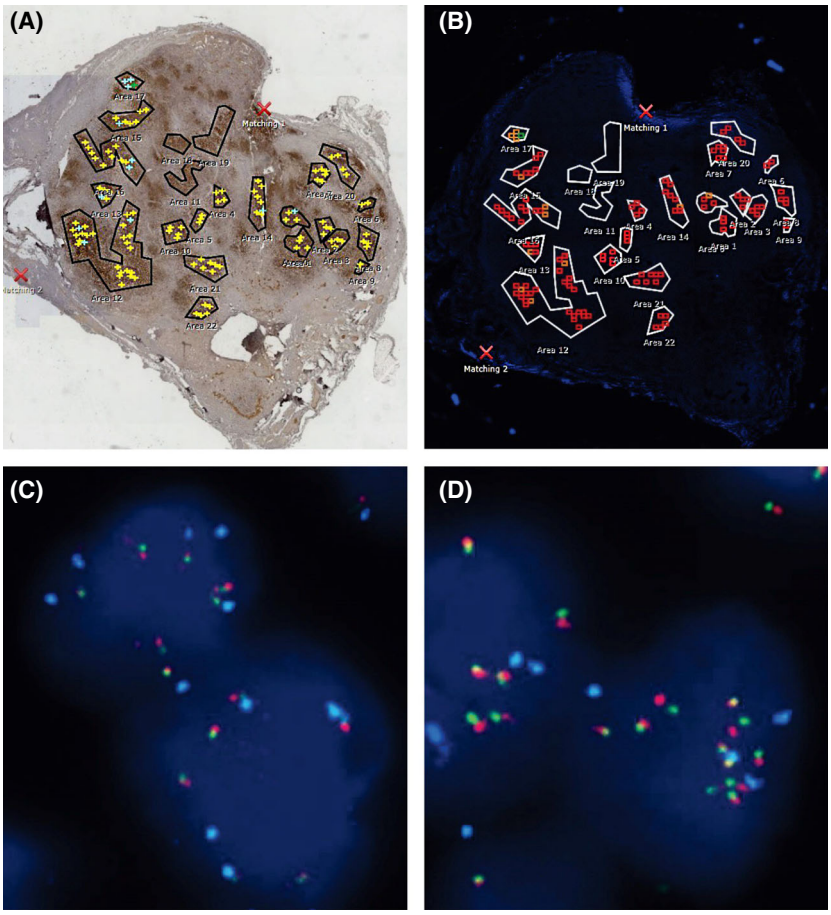

Fig 1. Combination of cluster of differentiation 30 (CD30) immunohistochemistry (IHC) and TriCheck fluorescence *in situ* hybridisation (FISH) using serial slides. After CD30 IHC, the slide was scanned and regions of interest containing large amounts of CD30 positive tumour cells were selected (A). Afterwards, FISH was performed and scanned using the 4′,6-diamidino-2-phenylindole (DAPI) filter. An 'overlay' was performed for transmission of the previously selected regions of interest (B) and 50 tumour cells in each case were analysed regarding their 9p24.1 status (C, D; ×60 Objective).

IHC analysis

The IHC analysis for PD-L1 and MHC-I/II was available for 75 and 81 patients respectively. In 55 (73%) cases, membranous PD-L1 expression was observed in the majority (>50%) of HRSC. Nine cases (12%) each expressed PD-L1 on 25–50% or <25% of HRSC respectively. Only two of the 75 evaluable cases (3%) displayed no PD-L1 expression in the neoplastic cells. We found a tendency towards a higher frequency of PD-L1 positivity ($HRSC^{+}$) among cases assigned to the 'amplified' CNA group (17/19; 89%) *versus* 38/56 (68%) in the 'copy gain' group ($P = 0{\cdot}078$; Fig 2C).

We observed membranous MHC-I expression in >50% of the tumour cells ($HRSC^{+}$) in only eight (10%) of the 81 evaluable cases, whereas we found membranous MHC-II expression in >50% of the tumour cells ($HRSC^{+}$) in 46 (57%) of the 81 evaluable cases. Two (2%) and eight (10%) cases showed a wide spectrum of membranous MHC-I and MHC-II expression respectively, ranging from negative to weakly positive to strongly positive, and were scored as undecided ($HRSC^{+/-}$). Membranous expression in <50% of the tumour cells was observed in 71 (88%) of cases for MHC-I and in 27 (33%) cases for MHC-II respectively; these cases were classified as $HRSC^{-}$. MHC-I and -II expression was similar among patients classified in the 'amplification' and 'copy gain' groups (Fig 2D, E; Table SII). The distribution of 9p24.1 CNA as well as the PD-L1, MHC-I/II IHC and Epstein–Barr virus (EBV) status per case are depicted in Fig 3.

Early response according to CNAs and PD-L1 expression

The interim staging after two cycles of N-AVD (concomitant treatment group) or four doses of nivolumab (sequential treatment group) was done in 78 of the 82 analysed patients; four patients dropped out before interim staging due to adverse events or violation of entry criteria.[17] All 40 analysed patients in the concomitant group and 36 (95%) out of 38 patients in the sequential group achieved objective response defined as either partial remission or complete remission (CR) in the interim staging (Table I). CR was observed in 37 (93%) and 21 (55%) patients respectively. We identified no apparent impact of 9p24.1 CNAs on early response, neither for CNA category [CR in 45 (76%) of 59 patients categorized as 'copy gain' vs. 13 (68%) of 19 patients with 'amplification'; $P = 0{\cdot}55$; Table I, Fig 4A; Table SIII] nor for PD-L1 protein expression [CR in 39 (72%) of 54 $HRSC^{+}$ patients vs. 12 (71%) of 17 patients with $HRSC^{<+}$; $P = 1{\cdot}00$; Fig 4B, Table SIV], MHC-I [CR in five (71%) of seven $HRSC^{+}$

Table I. Clinical characteristics and outcomes of the patients and pathohistological subtypes.

	Total (N = 109)			Analysed, treatment group		CNA category		
	Not analysed	Analysed	P	Concomitant treatment	Sequential treatment	Copy gain	Amplification	P
N	27	82		41	41	62	20	
Treatment group, n (%)								
Concomitant treatment	14 (52)	41 (50)	1·00	41 (100)	0	33 (53)	8 (40)	0·44
Sequential treatment	13 (48)	41 (50)		0	41 (100)	29 (47)	12 (60)	
Age, years, median (range)	24 (18–57)	28 (18–60)	0·036	27 (18–57)	29 (18–60)	28 (18–60)	30 (19–52)	0·77
Sex, n (%)								
Female	16 (59)	49 (60)	1·00	24 (59)	25 (61)	37 (60)	12 (60)	1·00
Male	11 (41)	33(40)		17 (41)	16 (39)	25 (40)	8 (40)	
Performance status, n (%)								
ECOG = 0	16 (59)	67 (82)	0·035	34 (83)	33 (80)	51 (82)	16 (80)	1·00
ECOG = 1	11 (41)	15 (18)		7 (17)	8 (20)	11 (18)	4 (20)	
Ann Arbor, n (%)								
IA	1 (4)	3 (4)	1·00	2 (5)	1 (2)	3 (5)	0	0·92
IB	0	1 (1)		0	1 (2)	1 (2)	0	
IIA	21 (78)	61 (74)		31 (76)	30 (73)	45 (73)	16 (80)	
IIB	5 (19)	17 (21)		8 (20)	9 (22)	13 (21)	4 (20)	
Risk factors, n (%) or n/N (%)								
Involvement of three or more nodal areas	20 (74)	55 (67)	0·63	30 (73)	25 (61)	39 (63)	16 (80)	0·18
Bulky disease[†]	21/26 (81)	51 (62)	0·098	21 (51)	30 (73)	39 (63)	12 (60)	1·00
Elevated ESR[††]	15 (56)	37 (45)	0·38	18 (44)	19 (46)	25 (40)	12 (60)	0·20
Extranodal disease	6 (22)	8 (10)	0·11	4 (10)	4 (10)	6 (10)	2 (10)	1·00
Large mediastinal mass[‡]	7 (26)	15 (18)	0·41	5 (12)	10 (24)	11 (18)	4 (20)	1·00
Histological subtype, n (%) or n/N (%)								
NSHL	9/26 (35)	60 (73)	<0·0001	31 (76)	29 (71)	45 (73)	15 (75)	0·85
MCHL	1/26 (4)	12 (15)		6 (15)	6 (15)	10 (16)	2 (10)	
LRHL	0	3 (4)		2 (5)	1 (2)	2 (3)	1 (5)	
Unspecified	16/26 (62)	7 (9)		2 (5)	5 (12)	5 (8)	2 (10)	
EBV LMP1, n (%) or n/N (%)								
Negative	20/23 (87)	62/71 (87)	1·00	33/37 (89)	29/34 (85)	45/53 (85)	17/18 (94)	0·43
Positive	3/23 (13)	9/71 (13)		4/37 (11)	5/34 (15)	8/53 (15)	1/18 (6)	
Early response[‡‡], n (%) or n/N (%)								
CR	15 (56)	58/78 (74)	0·090	37/40 (93)	21/38 (55)	45/59 (76)	13/19 (68)	0·55
PR	12 (44)	18 (23)		3/40 (8)	15/38 (39)	13/59 (22)	5/19 (26)	
No change	0	1 (1)		0	1/38 (3)	0	1/19 (5)	
Progressive disease	0	1 (1)		0	1/38 (3)	1/59 (2)	0	

CNA, copy number alteration; CR, complete remission; LMP1, latent membrane protein 1; LRHL, lymphocyte-rich subtype of classical Hodgkin lymphoma (cHL); MCHL, mixed cellularity subtype of cHL; NSHL, nodular sclerosis subtype of cHL; PR, partial remission.

[†]Presence of a lesion with ≥5 cm in greatest diameter.

[††]ESR: erythrocyte sedimentation rate; ≥50 mm/h for patients without B symptoms and ≥30 mm/h in case of B symptoms.

[‡]≥A third of the maximal thoracic diameter as measured on chest radiography.

[‡‡]After two cycles of nivolumab and doxorubicin, vinblastine, and dacarbazine (N-AVD) in the concomitant group and after four doses of nivolumab in the sequential group, respectively.

Fig 2. (A) Spectrum of 9p24.1 copy number alterations (CNAs). Each classical Hodgkin lymphoma (cHL) patient sample is represented by a column, and in each sample the percentage of tumour cells with 9p24.1 disomy (black), polysomy (light pink), copy gain (pink), and/or amplification (red) is shown on the *y*-axis. The asterisk marks the single patient in the sequential treatment group who showed progressive disease. (B) Percentage of disomic tumour cells grouped by CNA category. (C) Programmed cell death 1 ligand 1 (PD-L1) expression grouped by CNA category. (D) Major histocompatibility complex (MHC)-I expression grouped by CNA category. (E) MHC-II expression grouped by CNA category. **t*-test; [†]Fisher's exact test of $HRSC^{+}$ *versus* $HRSC^{<+}$. HRSC, Hodgkin- and Reed–Sternberg cells.

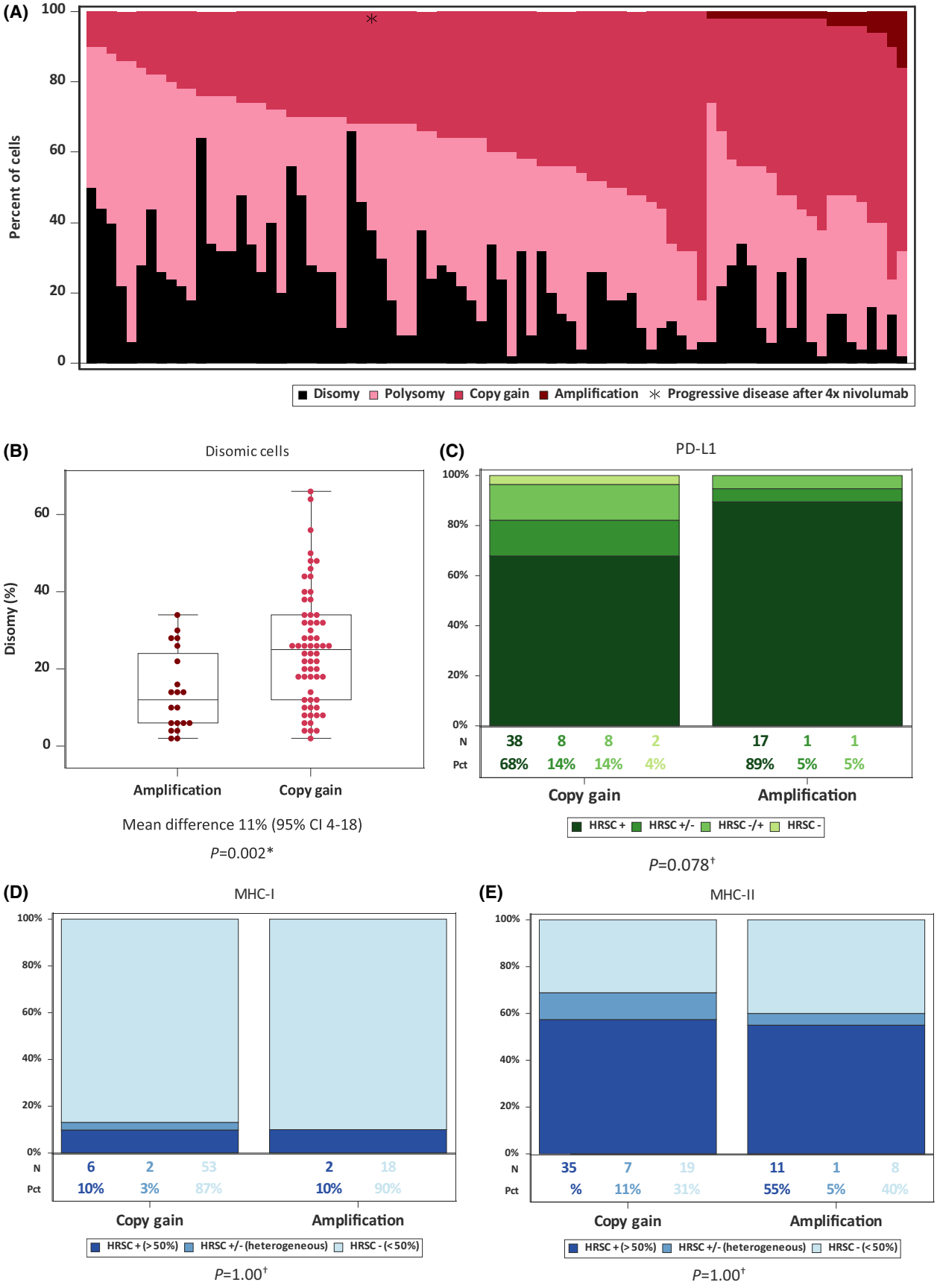

Table II. Copy number alterations by category.

	Copy gain CNA category	Amplification CNA category
Patients (N = 82)		
n (% of total N)	62 (76)	20 (24)
Positive cells/case, %		
Mean (SD)	35·1 (14·8)	3·9 (3·5)
Median (range)	32 (10–82)	2 (2–16)
PD-L1 copies/case, n		
Mean (SD)	3·9 (0·8)	4·9 (1·0)
Median (range)	3·8 (2·5–6·9)	4·7 (3·6–6·9)
Mean quotient PD-L1 (chromosome 9 per case)		
Mean (SD)	1·2 (0·1)	1·4 (0·2)
Median (range)	1·2 (1·0–1·5)	1·1 (1·1–1·9)
Additional alterations		
Disomy, n (%)	62 (100)	20 (100)
Mean (SD) %	25·4 (15·0)	14·1 (10·4)
Median (range) %	25 (2–66)	12 (2–34)
Polysomy, n (%)	62 (100)	20 (100)
Mean (SD) %	39·5 (14·6)	34·2 (12·9)
Median (range) %	41 (2–80)	35 (10–68)
Copy gain, n (%)	62 (100)	20 (100)
Mean (SD) %	35·1 (14·8)	47·8 (9·4)
Median (range) %	32 (10–82)	49 (24–66)

CNA, copy number alteration; PD-L1, programmed cell death 1 ligand 1; SD, standard deviation.

patients vs. 52 (74%) of 70 patients with HRSC$^{-/+}$; P = 1·00; Fig 4C, Table SV] or MHC-II [CR in 34 (77%)]of 44 HRSC^{+} patients vs. 23 (70%) of 33 patients with HRSC$^{-/+}$; P = 0·60; Fig 4D, Table SVI]. There was also no evident association of early CR with mean PD-L1 copy number of the HRSC [mean (SD) 4·1 (1.0) among 58 patients with CR vs. 4·2 (0.9) among those with non-CR; P = 0·7; Fig 4E] or mean proportion of residual disomic tumour cells mean (SD) 23·3 (14.2)% among 58 patients with CR vs. 20·7 (16.1)% among 20 non-CR patients; P = 0·5; Fig 4F]. Only one patient in the sequential treatment group showed histologically verified primary progressive disease (marked with an asterisk in Figs 2A and 3). This patient showed a mean PD-L1 copy number of 3·5, was placed in the 'copy gain' CNA category, and we observed a relatively low PD-L1 expression (<25% of HRSC^{+}) and membranous staining for MHC-I and -II in <50% of the neoplastic cells by IHC.

Discussion

In recent years, immune checkpoint blockade, e.g. with the anti-PD-1 antibodies nivolumab and pembrolizumab, has become an important mainstay of cHL treatment in the r/r setting, and genetically driven high PD-L1 expression has been reported to be predictive of an improved outcome in this setting.[20] We recently demonstrated very high efficacy of concomitant and sequential anti-PD-1 and AVD first-line treatment in patients with early stage unfavourable cHL in the phase II NIVAHL trial.[17] In the present study, we analysed genomic alterations of 9p24.1, the genomic locus of PD-L1/*CD274*, in pre-treatment tumour biopsies from adult patients with cHL enrolled in the NIVAHL trial, to determine the distribution and potential prognostic impact of 9p24.1 CNA in this cohort. The HRSC of all investigated samples showed to some extent gains of 9p24.1, confirming that gain of 9p24.1 chromosomal material is a characteristic genetic alteration of cHL also in early stage unfavourable disease.[3,20]

Interestingly, we observed a high variability of 9p24.1 CNA in different tumour cells of the same patient, which largely matches previous reports.[3,20,23] Although polysomy and copy number gains of 9p24.1 were found in the neoplastic cells of all investigated samples, we observed an overall smaller range and lower magnitude of 9p24.1 alterations, as well as fewer samples assigned to the 'amplification' category (applying the criteria defined by Roemer *et al.*[3,20]) compared to the previously published data in advanced-stage disease and

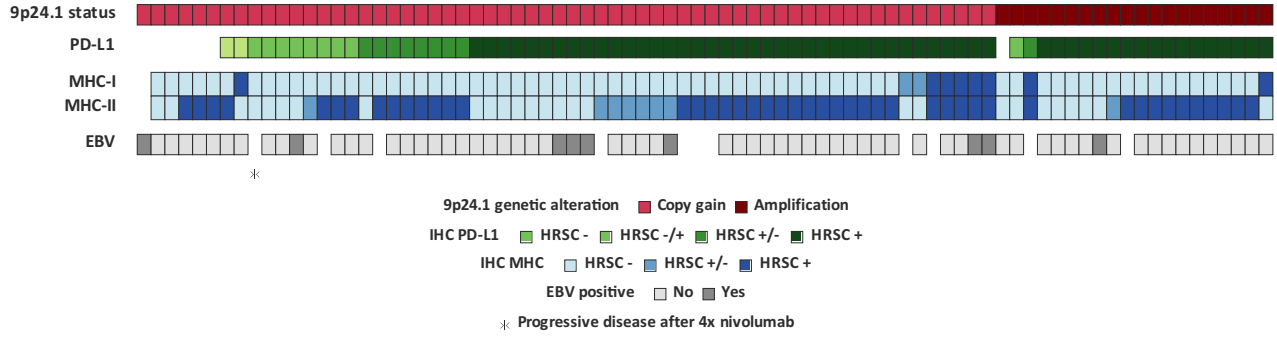

Fig 3. The heatmap depicts the distribution of 9p24.1 copy number alterations (upper row) as well as the programmed cell death 1 ligand 1 (PD-L1), major histocompatibility complex (MHC)-I, MHC-II immunohistochemistry and Epstein–Barr virus (EBV) status (from top down). PD-L1-phenotype groups: HRSC^{+}: >50% of tumour cells positive, HRSC$^{+/-}$: 25–50% of tumour cells positive, HRSC$^{-/+}$: <25% of tumour cells positive, HRSC^{-}: tumour cells negative. MHC-I and MHC-II immunohistochemistry groups: HRSC^{+}: membranous staining in >50% of the tumour cells, HRSC^{-}: membranous staining in <50% of the tumour cells. In few cases the tumour cells show a wide spectrum of membranous MHC expression, and were scored as undecided (HRSC$^{+/-}$). HRSC, Hodgkin- and Reed–Sternberg cells.

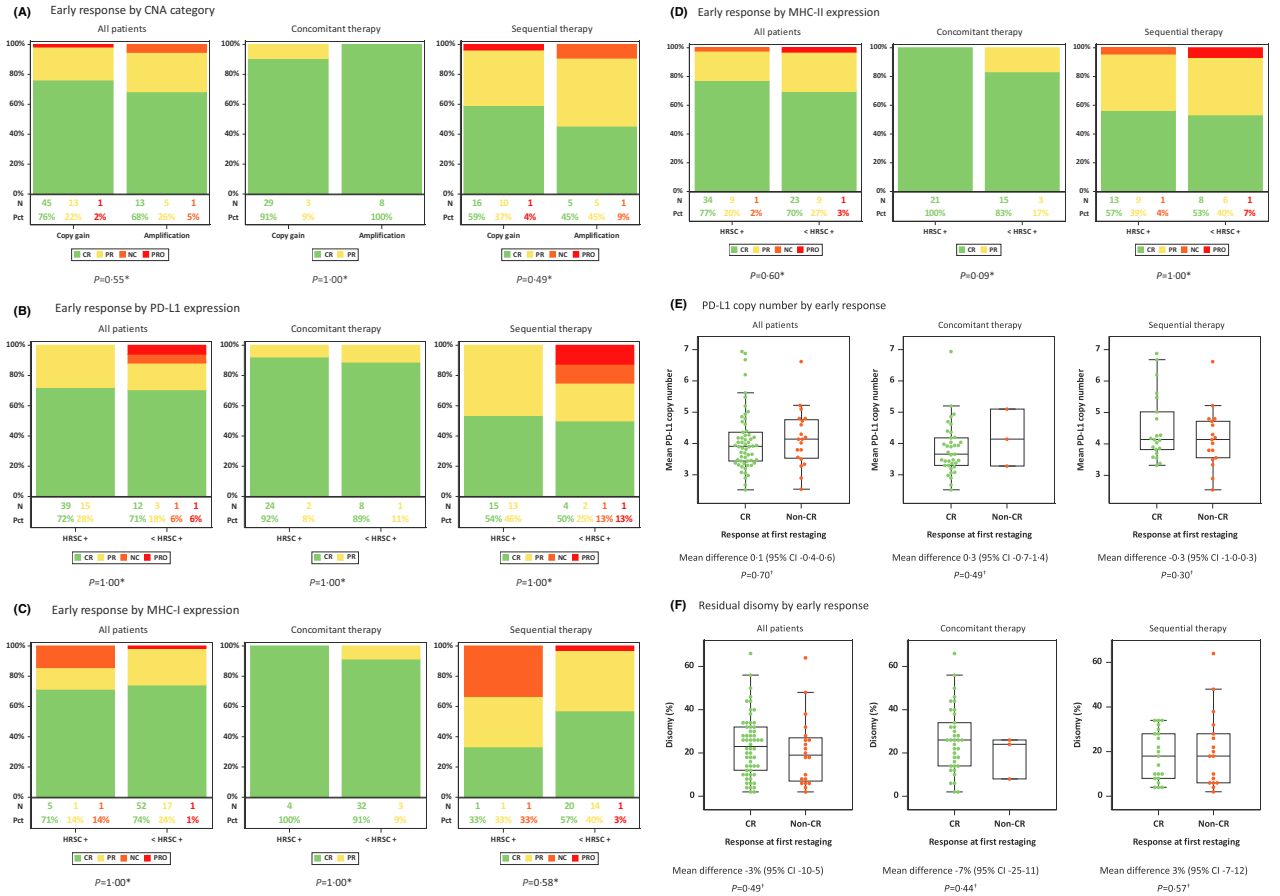

Fig 4. Early response [after two cycles of nivolumab and doxorubicin, vinblastine, and dacarbazine (N-AVD) in the concomitant group and after four doses of nivolumab in the sequential group, respectively] (A) by copy number alteration (CNA) category; (B) by programmed cell death 1 ligand 1 (PD-L1) expression; (C) by major histocompatibility complex (MHC)-I expression; (D) by MHC-II expression; (E) PD-L1 copy number by early response; and (F) by residual disomy by early response. Re-staging was not done due to early termination of protocol therapy in four of 82 patients (one of 42 with concomitant therapy and three of 41 with sequential therapy). $HRSC^{+}$: membranous staining in >50% of the tumour cells, $HRSC^{<+}$: all other cases. *Fisher's exact test of complete remission (CR) *versus* non-CR; †*t*-test of CR *versus* non-CR. HRSC, Hodgkin- and Reed–Sternberg cells.

the r/r setting.[3,18,20] Potential reasons for this might arise from the investigated patient cohorts: the NIVAHL trial investigated newly diagnosed early stage unfavourable cHL, while amplification of 9p24.1 has been reported to be more common in patients with cHL with advanced-stage disease and associated with shorter PFS with standard chemotherapy.[3] Our present findings may further support the notion from Roemer *et al.*[3] of higher level 9p24.1 CNA as an (initially) often subclonal event, which may confer an advantage to the afflicted tumour cell clone. Another explanation for the overall lower levels of 9p24.1 CNA might be the improved identification of tumour cells by our technical approach combining CD30 IHC and FISH compared to morphology alone,[3,18,20] as the selection of tumour cells in the FISH dark field solely based on morphology may lead to an analysis bias towards particular large tumour cells with likely more complex genetics.

High-magnitude 9p24.1 copy number gains/9p24.1 amplification and high PD-L1 expression have been suggested as potential predictors of a favourable outcome after PD-1 blockade with nivolumab in the r/r setting.[20] More recently, improved response rates to six cycles of N-AVD with higher PD-L1 expression were reported in newly diagnosed advanced-stage cHL, and a trend, although no significant association, towards more favourable responses to nivolumab monotherapy in patients with a higher PD-L1 expression of the HRSC has been reported.[18] Since in the NIVAHL trial the objective response and CR rates as well as the 1-year PFS were excellent, an analysis of 9p24.1 or PD-L1 expression towards these end-points is not feasible. Hence, we aimed to investigate the prognostic role of these parameters on the early response to either N-AVD or nivolumab monotherapy at first interim staging. However, neither 9p24.1 CNA category nor residual disomy, PD-L1, MHC-I, or MHC-II IHC had an apparent impact on early response in our present series, specifically if looking at the presumably in this regard most important subgroup with sequential treatment with initial nivolumab monotherapy. This discrepancy to the r/r

setting and to some extent also to advanced-stage cHL[18,20] may again arise from our presumably more favourable patient cohort, that is probably less likely to harbour prominent tumour cell clones with 9p24.1 amplification[3] and has an excellent treatment outcome, limiting the statistical power of our analysis. However, due to the overall small sample size and the explorative nature of our analyses, we cannot rule out an actual association of these parameters and response to treatment.

In our interpretation, our data point to further mechanisms beyond 9p24.1 gains/amplifications contributing to the observed extremely high efficacy of anti-PD-1 immune checkpoint blockade in cHL. These could include e.g. overall high mutational burden as suggested by Wienand *et al.*[24] or other complex and yet not well elucidated mechanisms of interaction between the HRSC and their accompanying immune cells.[25,26]

Classical HL characteristically show a high PD-L1 expression,[27–29] leading to its proposal as additional diagnostic marker for cHL.[30,31] Accordingly, we could identify only two cases (3%) without PD-L1 expression by IHC in the present cohort, and PD-L1 expression in >50% of the tumour cells was observed in 73% of patients. We observed a tendency towards a higher PD-L1 expression in cases with an increased copy number of the PD-L1 locus. This may point to further mechanisms beyond genomic 9p24.1 alterations underlying the high expression of PD-L1 on HRSC, such as EBV infection and constitutive activator protein 1 activity[32] as already suggested by Menter *et al.*[28] However, in our present series, only a minority of cases were EBV-positive and we could not observe any association between EBV infection and PD-L1 expression.

Moreover, we investigated the antigen presentation capacities of the neoplastic cells by B2M and HLA-DR/DP/DQ IHC as surrogates for MHC-I and -II respectively. Consistent with previous reports,[2,4,20] we observed diminished B2M expression of the neoplastic cells in 88% of the investigated samples, again underlining the notion, that in cHL response to PD-1 inhibition is mainly independent from the MHC-I/$CD8^+$ T-cell axis.[20,25,26] In contrast, MHC-II expression, which has been suggested as further potential predictor of a favourable outcome after PD-1 blockade with nivolumab in the r/r setting,[20] was retained in the majority of HRSC in 57% of the cases. Further analyses regarding a potential impact of MHC-II expression on outcome were limited by the excellent outcomes in our present cohort.

In conclusion, our present data further support the fact that 9p24.1 gains represent a characteristic genomic alteration of cHL in all stages but display a high intratumoral heterogeneity. Compared to the reported data from advanced-stage and r/r cHL, we detected an overall lower range and magnitude of 9p24.1 gains in the present investigated patient cohort with early stage unfavourable cHL, which may point to stage-dependent different tumour biology and/or to evolving tumour subclones. Moreover, we did not find an obvious impact of 9p24.1 copy number, MHC-I/-II or PD-L1 expression on early response to nivolumab-based first-line treatment in this patient cohort, with overall excellent clinical outcomes to anti-PD-1-based first-line therapy. Larger studies investigating an extended set of potential prognostic markers are required to identify robust parameters to optimally harness checkpoint inhibition in cHL.

Ethics approval and consent to participate

The investigator-sponsored GHSG NIVAHL trial including the correlative studies presented in the present study was approved by the Ethics Committee of the Faculty of Medicine of the University of Cologne. All patients of whom material was analysed in the present study provided written informed consent before enrolment into the study.

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Authors contributions

All authors contributed to the conception and design of the study. Study material and specimen were provided by Paul J. Bröckelmann, Susana Ben-Neriah, Stephanie Sasse, Sven Borchmann, Michael Fuchs, Peter Borchmann, Andreas Engert, Wolfram Klapper and Andreas Rosenwald. Collection and assembly of data were performed by Elena Gerhard-Hartmann, Helen Goergen, Paul J. Bröckelmann, Anja Mottok, Tabea Steinmüller, Johanna Grund, Sarah Reinke, Johanna Veldman, Arjan Diepstra and Wolfram Klapper. Data analysis was performed by Elena Gerhard-Hartmann, Helen Goergen, Paul J. Bröckelmann, Tabea Steinmüller, Johanna Grund, Alberto Zamò, Sarah Reinke, Johanna Veldman, Arjan Diepstra and Wolfram Klapper. The NIVAHL trial was led by Paul J. Bröckelmann and Andreas Engert. The present analysis was supervised by Wolfram Klapper and Andreas Rosenwald. The first draft of the manuscript was written by Elena Gerhard-Hartmann and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

Paul J. Bröckelmann reports grants from Bristol Myers Squibb, Merck Sharpe and Dohme, and Takeda; and personal fees and non-financial support from Bristol-Myers Squibb, Celgene and Takeda, all outside the submitted work. Sven Borchmann reports personal feed and non-financial support from Bristol-Myers Squibb and Takeda, all outside the submitted work. All other authors declare no potential conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplementary methods and results.

Table SI. Immunohistochemical antibody panel. Only membranous staining was considered positive.

Table SII. Immunohistochemistry (IHC) parameters by copy number alteration (CNA) category.

Table SIII. Early response by copy number alteration (CNA) category.

Table SIV. Early response by programmed cell death 1 ligand 1 (PD-L1) expression.

Table SV. Early response by major histocompatibility complex (MHC)-I expression.

Table SVI. Early response by major histocompatibility complex (MHC)-II expression.

Fig S1. *H*-Score-like fluorescence *in situ* hybridisation (FISH) analysis by copy number alteration (CNA) category.

Fig S2. *H*-Score-like fluorescence *in situ* hybridisation (FISH) analysis by early response.

Fig S3. Programmed death ligand 1 (PD-L1) expression of the bystander cells by early response.

Fig S4. Programmed death ligand 1 (PD-L1) digital image analysis [including Hodgkin- and Reed–Sternberg cells (HRSC) and bystander cells] by early response.

References

1. Connors JM, Cozen W, Steidl C, Carbone A, Hoppe RT, Flechtner HH, et al. Hodgkin lymphoma. *Nat Rev Dis Primers*. 2020;**6**:61.
2. Reichel J, Chadburn A, Rubinstein PG, Giulino-Roth L, Tam W, Liu Y, et al. Flow sorting and exome sequencing reveal the oncogenome of primary Hodgkin and Reed-Sternberg cells. *Blood*. 2015;**125**:1061–72.
3. Roemer MG, Advani RH, Ligon AH, Natkunam Y, Redd RA, Homer H, et al. PD-L1 and PD-L2 genetic alterations define classical hodgkin lymphoma and predict outcome. *J Clin Oncol*. 2016;**34**:2690–7.
4. Roemer MG, Advani RH, Redd RA, Pinkus GS, Natkunam Y, Ligon AH, et al. Classical Hodgkin lymphoma with reduced beta2M/MHC class I expression is associated with inferior outcome independent of 9p24.1 status. *Cancer Immunol Res*. 2016;**4**:910–6.
5. Steidl C, Shah SP, Woolcock BW, Rui L, Kawahara M, Farinha P, et al. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature*. 2011;**471**:377–81.
6. Armand P, Engert A, Younes A, Fanale M, Santoro A, Zinzani PL, et al. Nivolumab for relapsed/refractory classic Hodgkin Lymphoma after failure of autologous hematopoietic cell transplantation: extended follow-up of the multicohort single-arm phase II CheckMate 205 trial. *J Clin Oncol*. 2018;**36**:1428–39.
7. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*. 2015;**372**:311–9.
8. Younes A, Santoro A, Shipp M, Zinzani PL, Timmerman JM, Ansell S, et al. Nivolumab for classical Hodgkin's lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: a multicentre, multicohort, single-arm phase 2 trial. *Lancet Oncol*. 2016;**17**:1283–94.
9. Chen R, Zinzani PL, Lee HJ, Armand P, Johnson NA, Brice P, et al. Pembrolizumab in relapsed or refractory Hodgkin lymphoma: 2-year follow-up of KEYNOTE-087. *Blood*. 2019;**134**(14):1144–53.
10. Armand P, Shipp MA, Ribrag V, Michot JM, Zinzani PL, Kuruvilla J, et al. Programmed death-1 blockade with pembrolizumab in patients with classical Hodgkin lymphoma after brentuximab vedotin failure. *J Clin Oncol*. 2016;**34**:3733–9.
11. Armand P, Chen YB, Redd RA, Joyce RM, Bsat J, Jeter E, et al. PD-1 blockade with pembrolizumab for classical Hodgkin lymphoma after autologous stem cell transplantation. *Blood*. 2019;**134**:22–9.
12. Song Y, Gao Q, Zhang H, Fan L, Zhou J, Zou D, et al. Treatment of relapsed or refractory classical Hodgkin lymphoma with the anti-PD-1, tislelizumab: results of a phase 2, single-arm, multicenter study. *Leukemia*. 2020;**34**:533–42.
13. Shi Y, Su H, Song Y, Jiang W, Sun X, Qian W, et al. Safety and activity of sintilimab in patients with relapsed or refractory classical Hodgkin lymphoma (ORIENT-1): a multicentre, single-arm, phase 2 trial. *Lancet Haematol*. 2019;**6**:e12–e19.
14. Behringer K, Mueller H, Goergen H, Thielen I, Eibl AD, Stumpf V, et al. Gonadal function and fertility in survivors after Hodgkin lymphoma treatment within the German Hodgkin Study Group HD13 to HD15 trials. *J Clin Oncol*. 2013;**31**:231–9.
15. Borchmann S, Muller H, Haverkamp H, Baues C, Markova J, Huttmann A, et al. Symptomatic osteonecrosis as a treatment complication in Hodgkin lymphoma: an analysis of the German Hodgkin Study Group (GHSG). *Leukemia*. 2019;**33**:439–46.
16. Eichenauer DA, Thielen I, Haverkamp H, Franklin J, Behringer K, Halbsguth T, et al. Therapy-related acute myeloid leukemia and myelodysplastic syndromes in patients with Hodgkin lymphoma: a report from the German Hodgkin Study Group. *Blood*. 2014;**123**:1658–64.
17. Brockelmann PJ, Goergen H, Keller U, Meissner J, Ordemann R, Halbsguth TV, et al. Efficacy of nivolumab and AVD in early-stage unfavorable classic Hodgkin lymphoma: the randomized phase 2 German Hodgkin Study Group NIVAHL trial. *JAMA Oncol*. 2020;**6**:872–80.
18. Ramchandren R, Domingo-Domenech E, Rueda A, Trneny M, Feldman TA, Lee HJ, et al. Nivolumab for newly diagnosed advanced-stage classic Hodgkin lymphoma: safety and efficacy in the phase II CheckMate 205 study. *J Clin Oncol*. 2019;**37**:1997–2007.
19. Jeong AR, Ball ED, Goodman AM. Predicting responses to checkpoint inhibitors in lymphoma: are we up to the standards of solid tumors? *Clin Med Insights Oncol*. 2020;**14**. https://doi.org/10.1177/1179554920976366.
20. Roemer MG, Redd RA, Cader FZ, Pak CJ, Abdelrahman S, Ouyang J, et al. Major histocompatibility complex class ii and programmed death ligand 1 expression predict outcome after programmed death 1 blockade in classic Hodgkin lymphoma. *J Clin Oncol*. 2018;**36**:942–50.
21. Voltin CA, Mettler J, van Heek L, Goergen H, Muller H, Baues C, et al. Early response to first-line anti-PD-1 treatment in Hodgkin lymphoma: a PET-based analysis from the prospective, randomized phase II NIVAHL trial. *Clin Cancer Res*. 2021;**27**:402–7.
22. Nijland M, Veenstra RN, Visser L, Xu C, Kushekhar K, van Imhoff GW, et al. HLA dependent immune escape mechanisms in B-cell lymphomas: implications for immune checkpoint inhibitor therapy? *Oncoimmunology*. 2017;**6**:e1295202.
23. Mangano C, Ferrarini A, Forcato C, Garonzi M, Tononi P, Lanzellotto R, et al. Precise detection of genomic imbalances at single-cell resolution reveals intra-patient heterogeneity in Hodgkin's lymphoma. *Blood Cancer J*. 2019;**9**:92.

24. Wienand K, Chapuy B, Stewart C, Dunford AJ, Wu D, Kim J, et al. Genomic analyses of flow-sorted Hodgkin Reed-Sternberg cells reveal complementary mechanisms of immune evasion. *Blood Adv*. 2019;**3**:4065–80.
25. Cader FZ, Hu X, Goh WL, Wienand K, Ouyang J, Mandato E, et al. A peripheral immune signature of responsiveness to PD-1 blockade in patients with classical Hodgkin lymphoma. *Nat Med*. 2020;**26**:1468–79.
26. Reinke S, Brockelmann PJ, Iaccarino I, Garcia-Marquez MA, Borchmann S, Jochims F, et al. Tumor and microenvironment response but no cytotoxic T-cell activation in classic Hodgkin lymphoma treated with anti-PD1. *Blood*. 2020;**136**:2851–63.
27. Dilly-Feldis M, Aladjidi N, Refait JK, Parrens M, Ducassou S, Rullier A. Expression of PD-1/PD-L1 in children's classical Hodgkin lymphomas. *Pediatr Blood Cancer*. 2019;**66**:e27571.
28. Menter T, Bodmer-Haecki A, Dirnhofer S, Tzankov A. Evaluation of the diagnostic and prognostic value of PDL1 expression in Hodgkin and B-cell lymphomas. *Hum Pathol*. 2016;**54**:17–24.
29. Tanaka Y, Maeshima AM, Nomoto J, Makita S, Fukuhara S, Munakata W, et al. Expression pattern of PD-L1 and PD-L2 in classical Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, and gray zone lymphoma. *Eur J Haematol*. 2018;**100**:511–7.
30. Uccella S, Magnoli F, Vivian LF, Marchiori D, Leoni E, Tibiletti MG, et al. PD-1 and PD-L1 immunohistochemistry as a diagnostic tool for classic Hodgkin lymphoma in small-volume biopsies. *Am J Surg Pathol*. 2021;**45**:1293–6.
31. Volaric A, Bacchi CE, Gru AA. PD-1 and PD-L1 immunohistochemistry as a diagnostic tool for classic Hodgkin lymphoma in small-volume biopsies. *Am J Surg Pathol*. 2020;**44**:1353–66.
32. Green MR, Rodig S, Juszczynski P, Ouyang J, Sinha P, O'Donnell E, et al. Constitutive AP-1 activity and EBV infection induce PD-L1 in Hodgkin lymphomas and posttransplant lymphoproliferative disorders: implications for targeted therapy. *Clin Cancer Res*. 2012;**18**:1611–8.

