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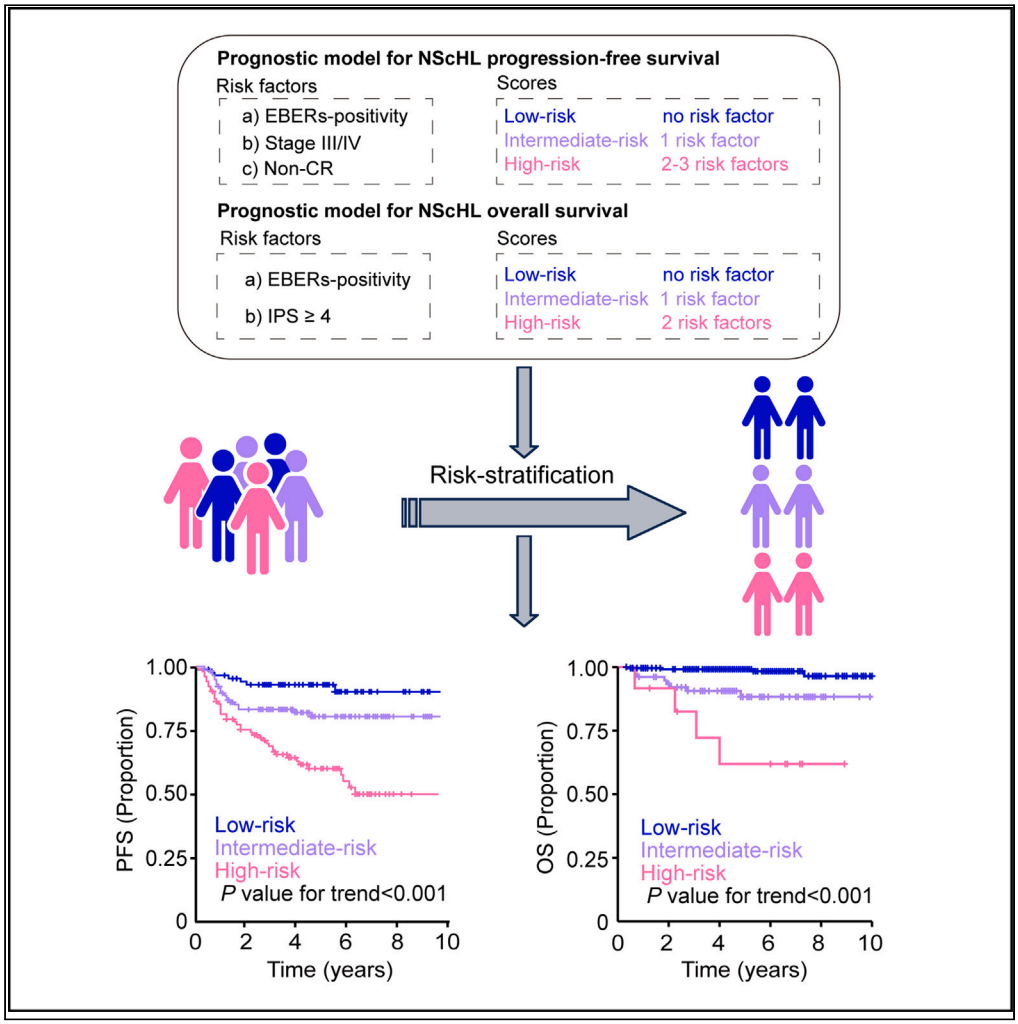

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Highlights

EBERs-positivity is an independent risk factor for both PFS and OS in NSCHL

EBERs combined with 1–2 clinical parameters successfully risk-stratified patients

Jiang et al., iScience *27*, 108630
January 19, 2024
https://doi.org/10.1016/j.isci.2023.108630

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Epstein-Barr virus-based prognostic model in nodular sclerosis classic Hodgkin lymphoma

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SUMMARY

The role of Epstein-Barr virus (EBV) in lymphoma cells of nodular sclerosis classic Hodgkin lymphoma (NScHL) is controversial. Our aim was to explore this and establish a clinically feasible model for risk stratification. We interrogated data from 542 consecutive subjects with NScHL receiving ABVD therapy and demonstrated EBV-infection in their lymphoma cells with EBV-encoded small RNAs (EBERs) *in situ* hybridization. Subjects were divided into training and validation datasets. As data from the training dataset suggested EBERs-positivity was the only independent prognostic factor for both progression-free survival (PFS) and overall survival (OS), we developed corresponding prognostic models based on it. Our models showed excellent performance in both training and validation cohort. These data indicate the close association of EBV infection and the outcomes of persons with NScHL receiving ABVD. Additionally, our newly developed models should help physicians estimate prognosis and select individualized therapy.

INTRODUCTION

Considerable data indicate that latent Epstein-Barr virus (EBV) infection drives malignant transformation in EBV-positive cHL.[1,2] 10–30 percent of people with nodular sclerosis classic Hodgkin lymphoma (NScHL) have evidence of EBV infection in the lymphoma cells at diagnosis defined by EBV-encoded small RNAs (EBERs) *in situ* hybridization (ISH) test-positivity.[2,3] However, the impact of EBERs expression in lymphoma cells on outcomes of people with NScHL is controversial.[3–9] The discrepancies in the results of previous studies may be attributed to the following reasons: (1) limited sample size; (2) mixed histological subtype investigated; (3) different treatment. Thus, a large systematic study focusing on particular histological subtype and treatment is required to shed light on this issue.

Although NScHL is generally associated with favorable disease outcomes, a subset of patients still progresses following standard treatment. Stratification of NScHL patients is therefore important for individual optimal management. Currently, patients receive stage-adapted treatment. For Ann Arbor stage I/II patients, patients would be allocated to defined groups on the presence or absence of clinical risk factors such as large mediastinal (bulky) mass, extranodal disease, elevated erythrocyte sedimentation rate (ESR), and involvement of three or more nodal sites.[10] For stage III/IV patients, the International Prognostic Score (IPS), which is defined by the number of adverse prognostic factors, was the most widely used prognostic tool for outcome estimation.[11] Unfortunately, this method for risk stratification has several limitations listed as follows: (1) the prognosis of early and advanced-stage patient needs to be estimated respectively. For early-stage ones, the presence of risk factors is considered, but for advanced-stage ones, IPS is used; (2) the IPS system has been used for over 25 years, the treatment strategy and the prognosis of cHL have been greatly improved, undermining its predictive efficacy for present patients.[12–14]

Besides, multiple studies used gene expression profiling to identify signatures that are associated with treatment outcomes.[15,16] High numbers of macrophages and non-malignant B-cells in the tumor microenvironment are associated with adverse and favorable prognosis, respectively.[17–19] However, neither gene expression profiling nor enumeration of cells have found their way into clinical practice. Many other

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https://doi.org/10.1016/j.isci.2023.108630

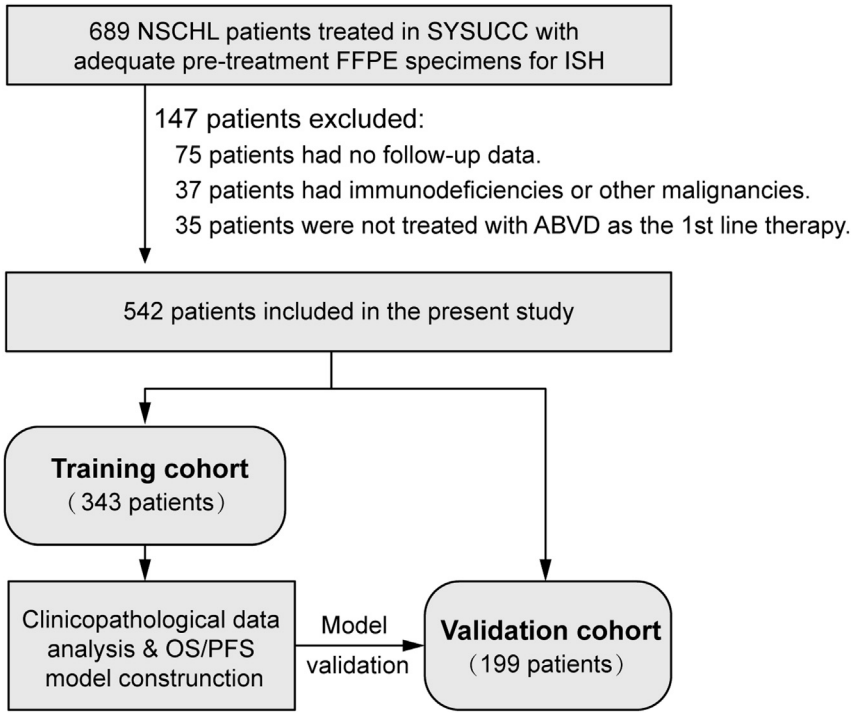

Figure 1. CONSORT flow diagram

tissue-based prognostic or predictive biomarkers in both lymphoma cells and the tumor microenvironment have suffered the same fate due to lack of validation or limited utility in clinical decisions.

In present study, we interrogated data from 542 consecutive subjects with NScHL receiving ABVD (doxorubicin, bleomycin, vinblastine, and dexamethasone) and used these data to determine the prognostic significance of EBV infection in NScHL. In addition, we combined EBERs with other clinical parameters to propose a clinically feasible prognostic models for progression-free survival (PFS) and overall survival (OS) and validate its value.

RESULTS

Subject clinical co-variates

Data from 542 consecutive subjects with NScHL receiving ABVD as initial therapy at Sun Yat-sen University Cancer Center from December, 2006 to December, 2019 was interrogated (CONSORT flow diagram; Figure 1). Subjects were divided by institute at the time of initial biopsy into training (N = 343) and validation (N = 199) datasets. The training dataset of 343 subjects includes 200 males (58%; Table S1). Median age is 27 years (Interquartile Range [IQR], 22–35 years). 178 (53%) were Ann Arbor stage-1/-2. 164 (48%) had B-symptoms and 269 (80%), mediastinal involvement. 160 (47%) received involved field radiation therapy. 17 (5.0%) patients with relapsed and refractory disease received autologous stem cell transplant (ASCT). 314 subjects received treatment response evaluation of whom 182 (58%) achieved a clinical complete remission (Table S1).

The validation dataset of 199 subjects includes 110 males (55%). Median age is 29 years (IQR:21–39 years). 114 (59%) were Ann Arbor stage-1/-2, 58 (38%) had B-symptoms and 148 (82%), mediastinal involvement (Table S1). 82 (50%) received involved field radiation therapy and 10, an autotransplant. 173 subjects were evaluated for response of whom 115 (66%) achieved a clinical complete response (Table S1).

There were no significant differences in clinical co-variates between the training and validation datasets except for B symptoms (Table S1). We also compared the baseline of clinical co-variates for cases diagnosed in earlier or later years and no significant difference was observed (Table S2).

EBERs expression in the training dataset

EBERs were detected in lymphoma cells in 80 subjects (23%) in the training dataset (Figure S1; Table 1). EBERs-test-positivity was significantly more common in males (30% versus 14%; p = 0.001) and varied with age with a bimodal distribution, a 1st peak in subjects <10 years of age and a 2nd in subjects >50 years (Figure S2A; Table 1). EBERs-test-positivity was correlated to advanced clinical stage (29% vs. 18%, p = 0.02), absence of mediastinal mass (33% vs. 21%, p = 0.04), IPS score >4 (48% vs. 25%, p = 0.02), elevated LDH level (30% vs. 19%, p = 0.02), WBC <15*10^{9}/L (26% vs. 12%, p = 0.03), ESR >50 mm/h (27% vs.16%, p = 0.04) and serum/plasma EBV DNA copy number >1000

Table 1. Co-variates in the training cohort

	EBERs			
		Negative (%; 95% CI)	Positive (%; 95% CI)	p-value
N	343	263 (77%)	80 (23%)	
Sex				
Male	200	140 (70% [62, 78%])	60 (30% [22, 38%])	0.001
Female	143	123 (86% [80,92%])	20 (14% [8, 20%])	
Age (years)				
≤10	12	3 (25% [1, 50%])	9 (75% [51, 100%])	<0.001
10–50	304	249 (82% [78, 86%])	55 (18% [14, 22%])	
>50	27	11 (41% [22, 59%])	16 (59% [41, 78%])	
Ann Arbor stage				
I/II	178	146 (82% [76, 88%])	32 (18% [12, 24%])	0.02
III/IV	155	110 (71% [64, 78%])	45 (29% [22, 36%])	
B-symptoms				
Yes	164	122 (74% [68, 81%])	42 (26% [19, 32%])	0.33
No	175	138 (79% [73, 85%])	37 (21% [15, 27%])	
≥10 cm mass				
No	229	176 (77% [71, 82%])	53 (23% [18, 29%])	0.20
Yes	25	22 (88% [75, 100%])	3 (12% [1, 25%])	
Mediastinal mass				
Yes	269	213 (79% [74, 84%])	56 (21% [16, 26%])	0.04
No	67	45 (67% [56, 78%])	22 (33% [22, 44%])	
Bone marrow involvement				
Yes	6	3 (50% [10, 90%])	3 (50% [10, 90%])	0.14
No	324	251 (77% [73, 82%])	73 (23% [18, 27%])	
Multiple sites				
No	84	62 (74% [64, 83%])	22 (26% [17, 36%])	0.82
Yes	121	91 (75% [68, 83%])	30 (25% [17, 33%])	
KPS				
<90	37	29 (78% [65, 92%])	8 (22% [8, 35%])	0.79
≥90	292	223 (76% [72, 81%])	69 (24% [19, 29%])	
IPS[a]				
≤3	126	94 (75% [67, 82%])	32 (25% [18, 33%])	0.02
≥4	25	13 (52% [32, 72%])	12 (48% [28, 68%])	
LDH				
Normal	206	167 (81% [76, 86%])	39 (19% [14, 24%])	0.02
Increased	137	96 (70% [62, 78%])	41 (30% [22, 38%])	
Serum albumin (g/L)				
≥40	210	162 (77% [71, 83%])	48 (23% [17, 29%])	0.74
<40	127	96 (76% [68, 83%])	31 (24% [17, 32%])	
WBC concentration (10E+9/L)				
≥15	58	51 (88% [80, 96%])	7 (12% [4, 21%])	0.03
<15	280	208 (74% [69–79%])	72 (26% [21–31%])	
Hemoglobin (g/dL)				
≥10.5	284	215 (76% [71, 81%])	69 (24% [19, 29%])	0.36
<10.5	54	44 (81% [71, 92%])	10 (19% [8.29%])	

(*Continued on next page*)

Table 1. *Continued*

	EBERs			
		Negative (%; 95% CI)	Positive (%; 95% CI)	p-value
Lymphocyte concentration (<0.6 x 10E+9 or <8%)				
Yes	13	11 (85% [65, 104%])	2 (15% [4, 35%])	0.49
No	325	248 (76% [72, 81%])	77 (24% [19, 28%])	
ESR (mm/h)				
≥50	98	72 (73% [65, 82%])	26 (27% [18, 35%])	0.04
<50	146	123 (84% [78, 90%])	23 (16% [10, 22%])	
EBV-DNA copy number/ml				
<1000	119	101 (85% [79, 91%])	18 (15% [9, 22%])	<0.001
≥1000	13 13	0 (0%)	13 (100%)	

CI, Confidence Interval; EBERs, EBV-encoded small RNAs; ESR, erythrocyte sedimentation rate; IPS, international prognostic score; ISH, *in situ* hybridization; KPS, Karnofsky performance score; LDH, lactic dehydrogenase; NScHL, nodular sclerosis classic Hodgkin lymphoma.
[a]IPS was calculated for stage III/IV disease.

copies/mL (100% vs. 15%, p < 0.001) (Table 1). In addition, compared with EBERs-negative subjects (146/238, 61%), EBERs-positive subjects had a lower complete remission (CR) rate (36/76, 47%, p = 0.03).

EBERs-positivity, PFS and OS

Result of univariable analyses of correlations between EBERs-test result and clinical and laboratory co-variates for PFS are displayed in Table 2. In multi-variable analysis EBERs-positivity (Hazard Ratio [HR] = 1.85 [1.14, 3.00]; p = 0.01), Ann Arbor stage-III/-IV (HR = 1.87 [1.14, 3.08]; p = 0.01) and no complete remission (HR = 2.33 [1.43, 3.80]; p = 0.001) were significantly correlated with worse PFS.

Table 2. Regression analyses of PFS in the training cohort

	Uni-variable			Multi-variable		
	HR	95% CI	*P*	HR	95% CI	*P*
EBERs-positive	2.29	1.47, 3.57	<0.001	1.85	1.14, 3.00	0.01
Age ≥18 years	0.54	0.31, 0.91	0.02	–	–	0.15
B-symptoms	1.17	0.75, 1.80	0.49	–	–	–
≥10 cm mass	0.89	0.32, 2.49	0.83	–	–	–
Mediastinal mass	1.18	0.73, 1.92	0.50	–	–	–
IPS score ≥4[a]	0.69	0.29, 1.62	0.39	–	–	–
Multiple nodes	1.48	0.84, 2.59	0.17	–	–	–
Bone marrow involvement	2.90	0.92, 9.22	0.07	–	–	–
Stage III/IV	2.24	1.40, 3.59	0.001	1.87	1.14, 3.08	0.01
Elevated LDH	1.46	0.95, 2.25	0.09	–	–	–
Albumin <40 g/dL	0.64	0.40, 1.04	0.07	–	–	–
Hemoglobin <10.5 g/dL	0.88	0.49, 1.55	0.88	–	–	–
ESR >50 mm/h	0.76	0.43, 1.34	0.34	–	–	–
WBC ≥15 × 10E+9/L	1.01	0.57, 1.80	0.96	–	–	–
Lymphocyte concentration (<0.6 x10E+9 or <8%)	0.59	0.15, 2.41	0.47	–	–	–
Deauville score (C2) > 3	1.64	0.81, 3.32	0.17	–	–	–
No complete remission	2.76	1.73, 4.39	<0.001	2.33	1.43, 3.80	0.001
No radiation therapy	0.84	0.55, 1.30	0.44	–	–	–

Abbreviations: CI, confidence interval; CR, complete remission; ESR, erythrocyte sedimentation rate; HR, hazard ratio; IPS, international prognostic score; LDH, lactic dehydrogenase; PFS, progression-free survival.
[a]IPS was calculated for stage III/IV.

Table 3. OS regression analyses of the training cohort

	Uni-variable			Multi-variable		
	HR	95% CI	*P*	HR	95% CI	*P*
EBERs-positive	4.24	1.67, 10.75	<0.01	4.44	1.11, 17.66	0.04
Age ≥18 years	2.68	0.37, 20.15	0.34	–	–	–
B-symptoms	1.52	0.58, 4.00	0.39	–	–	–
≥10 cm mass	0.04	0.00, 90.51	0.42	–	–	–
Mediastinal mass	1.34	0.39, 4.64	0.64	–	–	–
IPS score ≥4[a]	8.36	2.36, 29.67	0.001	6.17	1.69, 22.57	<0.01
Multiple nodes	1.58	0.41, 6.11	0.51	–	–	–
Bone marrow involvement	3.20	0.43, 24.17	0.26	–	–	–
Stage III/IV	1.94	0.70, 5.34	0.20	–	–	–
Elevated LDH	2.45	0.95, 56.32	0.06	–	–	–
Albumin <40 g/dL	2.09	0.83, 5.31	0.12	–	–	–
Hemoglobin <10.5 g/dL	1.14	0.33, 3.95	0.83	–	–	–
ESR >50 mm/h	2.66	0.89, 7.95	0.08	–	–	–
WBC ≥15 x 10E+9/L	1.37	0.45, 4.17	0.58	–	–	–
Lymphocyte concentration (<0.6 x 10E+9 or <8%)	3.22	0.82, 13.87	0.12	–	–	–
Deauville score (C2) > 3	1.46	0.36, 5.95	0.60	–	–	–
No complete remission	2.01	0.74, 14.02	0.19	–	–	–
No radiation therapy	4.43	1.28, 15.31	0.02	–	–	0.08

CI, confidence interval; CR, complete remission; ESR, erythrocyte sedimentation rate; HR, hazard ratio; IPS, International Prognostic Score; LDH, lactic dehydrogenase; OS, overall survival.
[a]IPS was calculated for stage III/IV.

Result of uni-variable analyses of correlations between EBERs-test result and clinical and laboratory co-variates for OS are displayed in Table 3. In multi-variable analyses EBERs-positivity (HR = 4.44 [1.11, 17.66]; p = 0.04) and IPS score ≥4 (HR = 6.17 [1.69, 22.57]; p < 0.01) were significantly correlated with worse OS.

Development of EBV-based prognostic models for PFS and OS

Next, based on EBERs-positivity, Ann Arbor stage-III/-IV and non-CR, the three significant variables identified in multi-variable analysis, we built a prognostic model for PFS in which no adverse variable constituted a low-risk cohort, 1, an intermediate-risk cohort and ≥2 a high-risk cohort (Figure 2A), representing 28%, 38% and 34% of subjects. Corresponding 5-year PFSs were 93% (87, 98%), 80% (73, 88%) and 60% (50, 70%; p-value for trend <0.001; Table 4; Figure 2B). With the low-risk cohort as reference corresponding HRs were HR = 2.4 (1.0, 5.6; p < 0.05) and HR = 6.0 (2.7, 13; p < 0.001; Table 4). The Area Under the Receiver-Operator Characteristic (AUROC) for predicting 5-year PFS is 0.69 (0.63, 0.79; Figure S3A).

Similarly, based on EBERs-positivity and IPS score ≥4, we built a prognostic model for OS in which no adverse variable constituted a low-risk cohort, 1, an intermediate-risk cohort and 2, a high-risk cohort (Figure 2A). 73%, 23%, and 4% of subjects were low-, intermediate or high-risk (Table 5). Corresponding 5-year OS were 99% (98,100%), 88% (81, 96%) and 62% (32, 92%; p-value for trend <0.001; Figure 2C; Table 5). With the low-risk cohort as reference corresponding HRs were HR = 6.3 (1.9, 21; p < 0.01) and 23 (5.7, 91; p < 0.001; Table 5). The AUROC for predicting 5-year OS is 0.73 (0.57, 0.90; Figure S3B).

Model validation

To validate accuracy of the model we interrogated a dataset of 199 subjects. The validation cohort had a similar sex and age and proportion of EBERs-test-positive subjects compared with the training cohort (Figure S2B). The model for PFS and OS were also successfully validated. Corresponding 5-year PFSs were 94% (87, 100%), 75% (62%, 88%) and 44% (27, 62%; P-value for trend <0.001; Table 4; Figure 2D). Corresponding 5-year OS were 98% (94,100%), 93% (84, 100%) and 50% (5, 95%; p-value for trend <0.001; Figure 2E; Table 5). The 5-year PFS model had a AUROC of 0.78 (0.70, 0.85; Figure S3C) and 5-year OS AUROC of 0.77 (0.57, 0.96; Figure S3D).

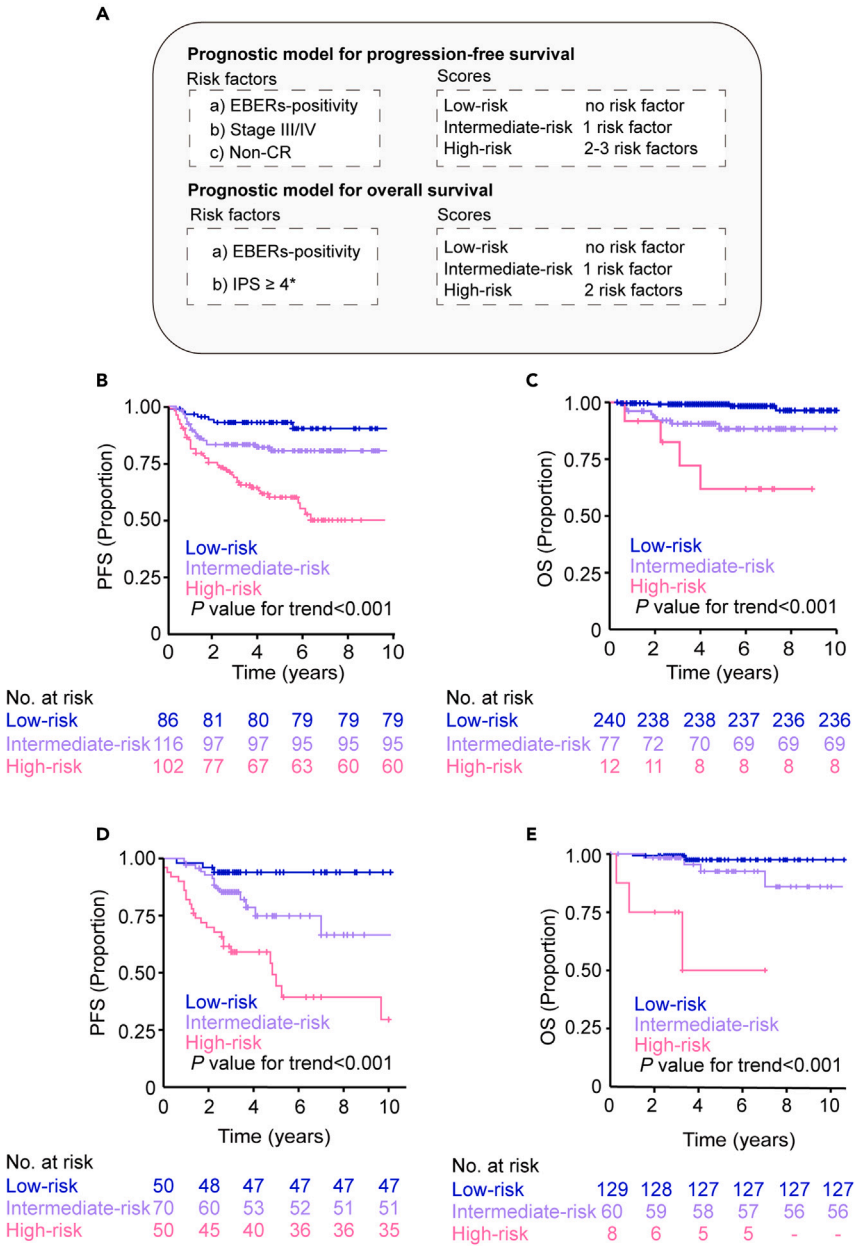

Figure 2. EBV-based prognostic model

(A) PFS and OS model; (B) K-M plot of PFS in the training cohort; (C) K-M plot of OS in the training cohort; (D) K-M plot of PFS in the validation cohort; (E) K-M plot of OS in the validation cohort. * IPS was calculated for stage III/IV disease. Subjects with stage III/IV disease, IPS ≥4 were considered to have this risk factor and others not.

Evaluation of EBV-based prognostic models in different age groups

As age is implicated as an important factor that influences patients' survival, we additionally evaluated the performance of our models in different age groups. All participants were classified as children (Age<18), young adults (Age>=18 & Age<26) or older adults (Age>=26).[20] Our results indicated that the low-risk, intermediate-risk, and high-risk patient groups in different age groups had statistically significant differences in progression-free survival rates, indicating that this model was suitable for evaluating the risk of disease progression among all age groups. (Figure S4A) For OS, our model performed well in adult patients aged 26 or above, but for patients aged under 26, the overall survival rates among different risk groups were not statistically significant, which might be attributed to better prognosis of NScHL in young patients and limited case number. (Figure S4B).

Table 4. PFS prognostic model

	N	Progression	5-year PFS	95% CI	OR	95% CI	p-value	HR	95% CI	p-value
Training Cohort[a]										
Low-risk (Ref.)	86(28%)	7	93%	87, 98%	1			1		
Intermediate-risk	116(38%)	21 (18%)	80%	73, 88%	2.5	1.0, 6.2	0.04	2.4	1.0, 5.6	<0.05
High-risk	102(34%)	42 (41%)	60%	50, 70%	8.0	3.3, 19	<0.001	6.0	2.7, 13	<0.001
Validation Cohort[a]										
Low-risk (Ref.)	50(29%)	3	94%	87, 100%	1			1		
Intermediate-risk	70(42%)	14 (20%)	75%	62, 88%	4.0	1.1, 15	0.03	3.5	1.0, 12	<0.05
High-risk	50(29%)	26 (52%)	44%	27, 62%	17	4.7, 62	<0.001	10	3.1, 34	<0.001

PFS, progression-free survival; OR, Odds Ratio; HR, Hazard Ratio; CI, confidence interval; Low-risk, 0 risk factor; Intermediate-risk,1 risk factor; High-risk, 2–3 risk factors.

[a]39 subjects in the training cohort and 29 in the validation cohort had missing values for model required parameters and were excluded.

DISCUSSION

Up to date, the prevalence and clinicopathological significance of EBV infection in NScHL has not been fully explored yet. Thus, a large systematic study is needed. We retrospectively investigated two large cohorts of patients diagnosed with NScHL and demonstrated the presence of EBV in around 25% of patients in South China. Our results showed that EBV-positive NScHL was more commonly found in male and advanced stage of disease. Interestingly, EBV infection rates varied with age, with the first peak occurred in patients less than 10 years of age and the second peak occurred in patients aged over 50. Coincidentally, in low- and middle-income countries, EBV is usually acquired at a young age and accordingly, EBV-positive cHL often occurs in childhood. The incidence of EBV-positive NScHL steadily increases with age due to the gradually declining immune system. In addition, our results revealed that EBV infection was correlated with adverse clinical parameters, including IPS score $\geq$4, elevated LDH level, WBC <15*10^9/L, ESR $\geq$50 mm/h, and a lower CR rate. Our results provided valuable evidence in the prevalence and clinicopathological significance of the EBV infection in this malignancy.

To date, the prognostic significance of EBV infection in cHL patients also remains unclear. Some studies have shown that EBV infection is a favorable prognostic factor,[9,21–27] while other studies have shown that it is an adverse prognostic factor.[4,28–30] These conflicting data have been usually attributed to the heterogeneous nature of the disease, discrepancies in the methods of EBV detection, and treatment regimens. Of note, most previously published studies included limited number of patients and mixed histological subtypes of cHLs.[3–6,31] The impact of EBV infection in people with NScHL is controversial and only studied in few subjects.[7,32] Of note, Myriam BD et al. reported that EBV infection only impacted the outcome of patients with NScHL, but not for other histotypes, such as mixed cellularity subtype. Concord results were also observed in our present study (Figure S5). EBERs-positivity had an adverse effect on the prognosis of NScHL patients. In addition, EBV-positive NScHL patients had a significant worse 5-year OS than EBV-negative patients (87% vs. 97%, p =0.001), as well as a worse 5-year PFS rate (62% vs. 81%, p < 0.001). EBERs-positivity was the only independent prognostic variable for both OS and PFS in NScHL patients treated with ABVD regimen. Therefore, our data indicate a substantial impact of EBV-infection assayed by EBERs-test-positivity on PFS and OS of persons with NScHL receiving ABVD.

ABVD chemotherapy is the most widely used frontline therapy for patients with NScHL at present. Since the prognosis of NScHL is generally favorable and is better than that of other types of cHL,[33] optimal management of patients with NScHL requires a precise diagnosis and risk assessment. Identification of the patients with high risk of progression or death allows for risk-adapted therapy to potentially increase the likelihood of cure. Currently, an accurate assessment of clinical stage is thought to be critical for the selection of appropriate therapy.

Table 5. OS prognostic model

Risk of Poor OS	N	Deaths	5-year OS	95% CI	OR	95% CI	P-value	HR	95% CI	p-value
Training Cohort[a]										
Low-risk (Ref.)	240(73%)	4	99%	98, 100%	1			1		
Intermediate-risk	77(23%)	8	88%	81, 96%	6.8	2.0, 24	<0.01	6.3	1.9, 21	<0.01
High-risk	12(4%)	4	62%	32, 92%	30	6.2, 140	<0.001	23	5.7, 91	<0.001
Validation Cohort[a]										
Low-risk (Ref.)	129(66%)	2	98%	94, 100%	1			1		
Intermediate-risk	60(31%)	5	93%	84, 100%	5.8	1.1, 31	0.02	4.0	0.8, 21	0.10
High-risk	8(3%)	3	50%	5, 95%	38	5.2, 282	0.001	41	6.6, 252	<0.001

OS, overall survival; OR, Odds Ratio; HR, Hazard Ratio; CI, confidence interval; Low-risk, 0 risk factor; Intermediate-risk,1 risk factor; High-risk, 2 risk factors.

[a]14 subjects in the training cohort and 2 in the validation cohort had missing values and were excluded.

Prognostic models that identify patients at low or high risk for recurrence, as well as the response to therapy as determined by PET scan, are used to optimize therapy.[34] However, unexpectedly, PET scan Deauville score after C2, which is widely used in clinical practice nowadays is not significantly related to patients' OS or PFS in the training cohort (Figure S6). This is interesting and worth further study. Possible reasons include: (1) patients with high Deauville score may switch to a more potent chemotherapy regimen to improve prognosis; (2) the prognosis of NSCHL patients is generally good. Considering the great need for risk stratification of NScHL in the clinic, high expense of PET scan and unclear association between Deauville score and prognosis, we developed PFS and OS prognostic models in the training dataset based on co-variates identified in Cox regression multi-variable analyses. EBERs-test result, stage and achieving a complete remission stratified subjects into three risk cohorts with 5-year PFSs of 93%, 80% and 60% (p-value for trend < 0.001). 5-year OS of the 3 cohorts based on EBERs-test result and International Prognostic Score (IPS) were 99%, 88%, and 62% (p-value for trend < 0.001). The AUROC for predicting the 5-year PFS and OS in the training cohort was 0.69 (95% CI: 0.63–0.79) and 0.73 (95% CI: 0.57–0.90), respectively. Results in the validation cohort had a AUROC for predicting the 5-year PFS and OS was 0.78 (95% CI: 0.70–0.85) and 0.77 (95% CI: 0.57–0.96), respectively. These prognostic models may help recognize patients who can be cured and who may not with ABVD regimen. For the later setting, optimizing therapeutic scheme should be considered to minimize the mortality and improve patient outcome. Therefore, different combinations of EBERs test result and other independent prognostic variable(s) identify different risk groups with different OS and PFS. These clinically feasible prognostic models may be useful for risk stratification and prognosis evaluation when making clinical decisions.

Considerable data suggest EBV is involved in the pathogenesis of some cases of cHL.[29] Genome-wide association studies report EBV-positive cHL is associated with genetic variants in the class I region (rs2734986, HLA-A; rs6904029, HCG9) whereas EBV-negative cHL is associated with rs6903608 in the class II region (rs6903608, HLA-DRA). Also differences in regulation of apoptosis and immune escape mechanisms between EBV-positive and -negative cHL are reported to correlate with angiogenesis.[4] These data suggest differences in genetics, biology, and clinical behavior in EBV-positive compared with -negative cHL. The recent development of EBNA1-inhibitors offered new therapeutic options for EBV-positive cHL.[35,36]

Compared with the previous work mentioned above,[7,32] our study had several major advantages here: 1st,we focused on NScHL patients with ABVD treatment, but in those work, all histological subtypes of cHL were involved which might introduce bias in prognosis evaluation. 2rd, both OS and PFS were calculated in our study and all patients investigated received ABVD regimen as the 1st line therapy. Treatment response was analyzed and adopted in our PFS model, allowing physicians to precisely risk-stratify patients during treatment period and tailor treatment intensity as needed, but in those work, information on treatment regimen and response was not provided. 3rd, our model is simple to use and can be applied in NScHL patients across stages and age, not focusing on a particular population.

In conclusion, our data indicate the substantial impact of EBV-infection in lymphoma cells on outcomes of persons with NScHL receiving ABVD. We also developed and validated prognostic models for PFS and OS. These data should help physicians estimate outcomes.

Limitations of the study

Our study has important limitations. First, it is retrospective with unavoidable biases. Second, the training and validation datasets were selected based on site of initial biopsy rather than randomization which may introduce a selection bias. Third, our conclusions and model apply only to subjects with NScHL receiving ABVD. 4th, although our sample was relatively large, validation with larger datasets receiving other therapies is needed including other races and ethnicities.

We also noticed that the complete remission rates calculated based our cohorts are lower than typically observed. We propose that this could be attribute to the different means of evaluating treatment response. As mentioned above, both CT and PET-CT were used for treatment response evaluation. In the training cohort, 160 and 154 patients received PET-CT and CT evaluation respectively, 117 patients (73.1%) were evaluated as CR with PET-CT but only 65 patients (42.2%) were considered as CR with CT. In the validation cohort, 79 patients (78.2%) were judged as CR among the 101 cases evaluated by PET-CT and 36 out of 72 patients were evaluated as CR by CT. As PET-CT evaluation is based on the metabolic activity rather than the volume of tumor, some patients may be considered as CR by PET-CT but not CT.

ADDITIONAL RESOURCES

None.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108630.

ACKNOWLEDGMENTS

RPG acknowledges support from the National Institute of Health Research (NIHR) Biomedical Research Centre. RPG and YL acknowledge support from the Ministry of Science and Technology of China (84000–51200002).

AUTHOR CONTRIBUTIONS

Concept, data analysis, typescript preparation: C.J.; data collection: L.Y.H., J.H.Z., Y.Y.H., Q.T.H., Q.Y., Y.W., and M.Z.; figures: S.L.; EBERs ISH: J.C.F.; typescript revision: Z.M.L., S.M.H., and R.P.G.; Design and supervision: Y.L., J.P.Y., and Y.H.H.

DECLARATION OF INTERESTS

R.P.G. is a consultant to NexImmune Inc. Nanexa Pharma Ascentage Pharm Group and Antengene Biotech LLC, Medical Director of FFF Enterprises Inc.; Partner in AZAC Inc.; Board of Directors of Russian Foundation for Cancer Research Support and Scientific Advisory Board: StemRad Ltd.

Received: July 5, 2023
Revised: September 23, 2023
Accepted: November 30, 2023
Published: December 4, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
FFPE tissue from NScHL patients	Sun Yat-sen University Cancer Center	N/A
Critical commercial assays		
EBV Probe ISH Kit	Zhongshan Golden Bridge Biotechnology Co. Ltd.	Cat#ISH-6021
Deposited data		
Raw data	This paper	RDDA2023682715
Software and algorithms		
SPSS v27.0	IBM	https://www.ibm.com/products/spss-statistics

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yuhua Huang MD, Ph.D. (huangyh@sysucc.org.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

(1) Raw data derived from medical records have been deposited at Research Data Deposit, and accession number is listed in the key resources table. Local law prohibits depositing raw datasets derived from patients outside of the country of origin. The dataset is available upon request if access is granted. To request access, please contact the lead contact.
(2) This paper dose not report original code.
(3) Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

We interrogated data from 542 consecutive subjects with NScHL in this study. The flowchart delineating process of model construction and our data analysis and the detail of these participants is presented in Figure 1 and Table 1 respectively.

METHOD DETAILS

Study population and sampling

We enrolled 542 consecutive subjects with NScHL receiving ABVD as initial therapy at Sun Yat-sen University Cancer Center from December, 2006 to December, 2019 (CONSORT flow diagram; Figure 1). Subjects were divided by institute at the time of initial biopsy into training (N = 343) and validation (N = 199) datasets. Diagnoses of all patients were confirmed by 2 experienced hematopathologists using the 2017 WHO Classification criteria.[37] Inclusion criteria included: (1) No documented prior immune deficiency disorder or cancer; 2) adequate pre-treatment formalin fixed paraffin embedded (FFPE) specimen for EBERs ISH detection; (3) ABVD as initial therapy; (4) available follow-up data. Subjects with Ann Arbor stage $\leq$2 received 4 cycles of ABVD with involved field radiation therapy of 24–45 Gy whilst those with Ann Arbor stage $\geq$3 began with ABVD and could receive other regiment such as BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone; 29 cases), GVD (gemcitabine, vinorelbine, pegylated liposomal doxorubicin; 23 cases), GND (gemcitabine, navelbine, doxorubicin; 14 cases) or brentuximab vedotin (7 cases) based on risk stratification or ABVD treatment response. Therapy response was evaluated by PET-CT or CT.[10–13] The Institutional Review Board of Sun Yat-sen University Cancer Center approved the study (SL-B2022-024-01) and informed consent form.

EBERs *in situ* hybridization test

An EBERs *in situ* hybridization test was performed to detect EBV infected lymphoma cells with the EBV Probe ISH Kit (ISH-6021, Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) according to the manufacture's protocol in FFPE specimens.[38] A positive test was

brownish-yellow nuclear staining in lymphoma cells (Hodgkin and Reed-Sternberg cells) and included a positive control in all experiments. Results were evaluated by two hematopathologists blinded to the clinical data.

Quantification of serum EBV DNA copy number

The method for EBV DNA quantification was according to previously published literature.[39] In brief, DNA was isolated using the QIAamp Blood Mini Kit (QIAgen, Inc., Valencia, CA, USA). The real-time quantitative PCR system was developed toward the BamHI-W region. The designs of amplification primers were as previously reported. The assay was performed with Roche LightCycler 480 with 40 cycles of reaction. β-globin served as the internal control and water blanks were included as negative control for each analysis. The exact copy number of EBV DNA was calculated from a calibration curve.

Clinicopathological information collection from medical records

Clinicopathological co-variates at diagnosis and survival data were respectively collected by two independent researchers blinded to each other. Discordances were adjudicated by a 3rd researcher. Co-variates considered included: (1) sex; (2) age; (3) Ann Arbor stage; (4) B-symptoms; (5) Karnofsky performance score (KPS); (6) longest diameter of the largest mass measured in transverse and coronal axes on pretherapy imaging; (7) mediastinal involvement; (8) involvement of ≥ 3 lymph node sites; (9) serum lactic dehydrogenase (LDH) and albumin concentrations; (10) hemoglobin, WBC and lymphocyte concentrations; (11) erythrocyte sedimentation rate (ESR); and (12) serum/plasma EBV-DNA copy number measured by real-time quantitative polymerase chain reaction. The International Prognostic Score (IPS) was calculated for subjects with Ann Arbor stage ≥ 3. Data on therapy, Deauville score on [^{18}F]-FDG after 2 ABVD courses and follow-up data were also collected. Therapy response was assessed by CT and/or PET as described.[40,41]

QUANTIFICATION AND STATISTICAL ANALYSIS

PFS and OS were calculated using the Kaplan-Meier method. Associations between EBERs-positivity and clinical co-variates were calculated with the Chi-square test. Uni- and multi-variable analyses were used to calculate the hazard ratio (HR) and corresponding 95% Confidence Interval (95% CI) with the Cox proportional hazards regression model. Co-variates significant at $p < 0.05$ in uni-variable analyses were included in multi-variable analyses. A forward stepwise approach was used to select co-variate $p < 0.05$ to use in model building. Odds (OR), Hazard Ratios (HR) and Area Under the Receiver-Operator Characteristic (AUROC) curve were calculated. Statistical analyses used SPSS software version 27.0 (Armonk, NY, USA).