Dynamic change in Epstein-Barr virus DNA predicts prognosis in early stage natural killer/T-cell lymphoma with pegaspargase-based treatment: long-term follow-up and biomarker analysis from the NHL-004 multicenter randomized study

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Abstract

The multi-center randomized phase III NHL-004 study compared etoposide, dexamethasone and pegaspargase (ESA) *versus* the methotrexate, etoposide, dexamethasone and pegaspargase (MESA) regimen, combined with sandwiched radiotherapy, in newly diagnosed early-stage nasal natural killer / T-cell lymphoma (NKTCL). Here we report the long-term outcomes (median follow-up, 64 months) and biomarker analysis. A total of 256 eligible patients aged 14-70 years were randomly assigned (1:1) to the ESA or the MESA arm. The 5-year progression-free survival (PFS) rates were 80.3% and 74.9% in the ESA and MESA arms (hazard ratio [HR]=0.78 [95% CI: 0.46-1.33], *P*=0.371), and the 5-year overall survival (OS) rates were 85.1% and 80.9% (HR=0.74 [95% CI: 0.40-1.37], *P*=0.332), respectively. No new safety signals related to treatments were observed. Interim plasma Epstein-Barr virus (EBV) DNA positivity and stable disease / progressive disease response were independent predictors of inferior PFS and OS. No prognostic significance was observed according to molecular subtypes. Interim EBV DNA positivity correlated with up-regulated chromatin remodeling alterations, immune escape-related genes, and decreased infiltrating monocytes / M1 macrophages. With low toxicity, non-intravenous administration, and an outpatient design, ESA with sandwiched radiotherapy achieved long-term durable response in patients with newly diagnosed early-stage NKTCL. Dynamic monitoring of plasma EBV DNA provided a clinical rationale for future mechanism-based therapy in NKTCL.

Introduction

Natural killer / T-cell lymphoma (NKTCL) is the most aggressive subtype of mature T-cell lymphomas and is closely related to Epstein-Barr virus (EBV) infection. Nearly 80% of early-stage nasal patients need combined-modality therapy.[1,2] The asparaginase-containing regimens plus radiotherapy have been proven effective and safe. First-line regimens like DeVIC (dexamethasone, etoposide, ifosfamide and carboplatin), VIDL (etoposide, ifosfamide, dexamethasone, L-asparaginase and cisplatin), GELOX/P-GEMOX (gemcitabine, L-asparaginase/pegaspargase and oxaliplatin), and DDGP (dexamethasone, cisplatin, gemcitabine, and pegaspargase) have been investigated over recent decades in early-stage NKTCL.[3-9] However, long-term survival data have been relatively limited. Regarding molecular alterations, three molecular subtypes were previously identified, including the TSIM (based on mutations in JAK-STAT pathway and *TP53*, as well as amp9p24.1/JAK2 locus, amp17q21.2/STAT3/5B/5A locus, amp9p24.1/PD-L1/2 locus, and del6q21), MB (based on *MGA* mutation and 1p22.1/BRDT LOH), and HEA (based on *HDAC9*, *EP300*, and *ARID1A* mutation) subtypes.[10] EBV infection correlated with poor clinical outcome in NKTCL through modulating immune-related oncogenic signaling and the tumor microenvironment.[11]

We previously reported the results of the multicenter randomized phase III NHL-004 study at the 2-year follow-up.[12] The NHL-004 trial compared the etoposide, dexamethasone and pegaspargase (ESA) regimen *versus* the methotrexate, etoposide, dexamethasone and pegaspargase (MESA) regimen, combined with sandwiched radiotherapy, in newly diagnosed early-stage nasal NKTCL. The ESA arm achieved a high overall response rate (ORR), 2-year progression-free survival (PFS) rate, and 2-year overall survival (OS) rate of 88.8%, 85.5%, and 92.0%, respectively, which were non-inferior to the MESA arm rates of 86.2%, 79.7%, and 84.6%. ESA with sandwiched radiotherapy was an effective regimen, with low toxicity, and convenient non-intravenous design. Here we report the updated, long-term results of the trial with a median follow-up of 64 months. In addition, biomarker analysis was performed to investigate the roles of dynamic plasma EBV DNA detection and efficacy assessment during treatment in this prospective study, as well as genomic and transcriptomic features.

Methods

Study design, patients and procedures

The NHL-004 study (clinicaltrials.gov identifier 02631239) was an open-label randomized phase III trial, which recruited patients from 27 hospitals of the Multicenter Hematology-Oncology Programs Evaluation System (M-HOPES) in China. Patients (aged 14-70 years) with histologically confirmed, newly diagnosed NKTCL, Eastern Cooperative Oncology Group (ECOG) performance status 0-2, Ann Arbor

stage IE-IIE, and a life expectancy of at least six months, were eligible. The study was approved by the institutional review boards of all centers and conducted in compliance with the principles of the Declaration of Helsinki. Informed consent was obtained from all enrolled patients.

Eligible patients were randomly assigned 1:1 (computer-assisted permuted-block randomization with a block size of 4) to receive 4 cycles of ESA (pegaspargase 2,500 IU/m^2 intramuscularly on day 1, etoposide 200 mg orally and dexamethasone 40 mg orally on days 2-4) or the MESA (methotrexate 1 g/m^2 intravenously on day 1, etoposide 200 mg orally and dexamethasone 40 mg orally on days 2-4 and pegaspargase 2,500 IU/m^2 intramuscularly on day 5) regimen, in combination with sandwiched radiotherapy (50 Gy) following the guidelines of the International Lymphoma Radiation Oncology Group.[13] The primary endpoint was ORR at the end of treatment (complete remission [CR] and partial remission [PR]), according to the Lugano 2014 criteria.[14] The secondary endpoints were 2-year PFS rate, 2-year OS rate, and toxicity (assessed according to CTCAE 4.0). Details of procedures, radiotherapy and assessment were as previously described.[12]

Biomarker analysis

Long-term survival of subgroups characterized by gender, age, ECOG, B symptoms, Ann Arbor stage, local tumor invasion, serum lactic dehydrogenase (LDH), Ki-67, International Prognostic Index (IPI),[15] Prognostic Index of Natural-Killer lymphoma-Epstein-Barr virus (PINK-E),[16] Chinese Southwest Oncology Group and Asia Lymphoma Study Group ENKTL system (CA)[17] and the Nomogram-revised Risk Index (NRI)[18] were analyzed. Dynamic plasma EBV DNA levels were detected before treatment, after 2 cycles, and at the end of treatment. Real-time fluorescent polymerase chain reaction (PCR) was used to detect plasma EBV DNA using Detection Kit for Epstein-Barr virus Nucleic Acid (PCR-Fluorescence Probing) (DaAnGene, Guangzhou, China) at all institutions. Any detectable load of EBV DNA was defined as positive. Interim response was assessed according to the same criteria as final response. Staining for MYC expression (N=149) was quantified by every 5% in pre-treatment tumor samples. CD56 was negative in 13% of patients. We further performed whole genome / exome sequencing (WGS/WES) (N=85), targeted DNA-sequencing (N=43), and RNA-sequencing (RNA-seq) (N=87) on available qualified pre-treatment tumor samples. The biomarker population is listed in *Online Supplementary Table S1*. There were no significant differences in clinical features or outcomes between the biomarker cohort and the entire cohort (*Online Supplementary Table S2*). Molecular subtypes were categorized by gene mutations and expression.[10] Gene Set Enrichment Analysis was used to identify the functional pathways. Proportions of immune cells were quantified by the CIBERSORTx method (https://cibersortx.stanford.edu).

Statistical analysis

Final follow-up was performed in April 2024. The efficacy and safety analyses were performed on the modified intent-to-treat (mITT) population, including all patients treated with at least one cycle of the ESA or the MESA regimen. Sample size calculation and amendments were as previously described.[12] Descriptive statistics were used to summarize patients' characteristics. Survival outcomes were calculated using the Kaplan-Meier method and survival curves were compared by the log-rank test. HR and associated 95% confidence interval were estimated using univariate Cox proportional-hazards model. Multivariate analysis was performed using multivariate Cox proportional-hazards model. Fisher's exact test was used to analyze the association of parameters with interim EBV DNA. The *t* test was used for comparison of gene expression profiles in interim EBV DNA positive and negative patients. Two-sided $P<0.05$ was considered significant. Data analyses and figure generation were performed using R 4.4.1 and SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

Results

Long-term outcomes

From March 16, 2016, to July 17, 2020, a total of 248 patients were treated with one of the assigned treatments (ESA [N=125] or MESA [N=123]) and these made up the mITT population (*Online Supplementary Figure S1*).[12] The median age of the ESA arm and MESA arm were 50 (range, 14-70) years and 46 (15-70) years. One hundred (80.0%), 11 (8.8%), 3 (2.4%), and 7 (5.6%) patients in the ESA arm achieved CR, PR, stable disease (SD), and progressive disease (PD), while the rates were 97 (78.9%), 9 (7.3%), 2 (1.6%), and 11 (8.9%) patients in the MESA arm, respectively (Table 1). The ORR in the ESA and MESA arms were similar: 88.8% *vs*. 86.2%, respectively. Results for the primary endpoint have been described previously.[12]

At the median follow-up of 64 months, 5-year PFS rates in the ESA and MESA arms were 80.3% (95% CI: 73.4-87.7%) and 74.9% (95% CI: 67.5-83.2%), respectively, with no statistical difference (hazard ratio [HR], 0.78; 95% CI: 0.46-1.33; P=0.371) (Figure 1A). The 5-year OS rates in the ESA and MESA arms were 85.1% (95% CI: 79.0-91.8%) and 80.9% (95% CI: 74.2-88.3%), respectively (HR, 0.74; 95% CI: 0.40-1.37; P=0.332) (Figure 1B). Median duration of response was not reached in either arm. Progression during the follow-up after first-line treatment was similar in the two arms (ESA *vs*. MESA: 11.2% *vs*. 9.8%) (Table 1).

Safety

The safety in both arms was consistent with the reported analysis, and no new safety signals were detected. There was one patient in each arm with second cancers: esophageal squamous cell carcinoma at ten months in the ESA arm

and tongue squamous cell carcinoma at four years in the MESA arm after anti-lymphoma treatments. Deaths from lymphoma occurred similarly: ESA (N=13); MESA (N=15). There were 5 deaths from other causes in the ESA arm *versus* 8 in the MESA arm.

Prognostic factors

Subgroups characterized by age, gender, ECOG, B symptoms, Ann Arbor stage, local tumor invasion, LDH, pre-treatment EBV DNA, Ki-67, IPI, PINK-E, CA, and NRI showed similar PFS results in the mITT population. Those with elevated LDH levels showed superior PFS in the ESA arm than the MESA arm (HR, 0.27; 95% CI: 0.09-0.81) (Figure 1C).

There were 45.6% and 47.2% of patients who presented with positive pre-treatment EBV DNA in the ESA and MESA arms. The proportion decreased to 8% and 13% after 2 cycles, respectively. At the end of treatment, 5.6% and 10.6% of patients had positive EBV DNA. As for interim response, 81 (64.8%), 30 (24.0%), 6 (4.8%), and 4 (3.2%) patients in the ESA arm achieved CR, PR, SD, and PD, respectively, while these rates were 68 (55.3%), 36 (29.3%), 9 (7.3%), and 6 (4.9%) patients in the MESA arm (Table 2).

Univariate analyses for PFS and OS were performed. Pre-treatment EBV DNA positivity was significantly related to inferior OS (P=0.035), while interim- and post-treatment EBV DNA positivity predicted both inferior PFS (both P<0.001) and OS (P<0.001 and P=0.007, respectively) (Figure 2). Of note, patients with interim response of SD and PD (after 2 cycles) indicated inferior PFS and OS, while prognosis of interim PR was similar with that of interim CR. As for final response (after 4 cycles), patients with PR, SD, and PD predicted poorer prognosis than those with CR. Interim EBV DNA positivity correlated with worse interim response (PR, SD, and PD, P=0.030), and post-treatment EBV DNA positivity correlated with worse final response (PR, SD, and PD, P=0.003) (*Online Supplementary Figure S2*).

Multivariate analysis was performed separately for factors at baseline, interim treatment and post-treatment. During treatment, both interim EBV DNA positivity and poor interim response (SD and PD) were independent adverse factors of PFS and OS. At the end of treatment, poor final response (PR, SD, and PD) predicted inferior PFS and OS, and post-treatment EBV DNA positivity predicted inferior PFS independently (Table 3).

High pre-treatment and interim EBV DNA levels were significantly associated with poor final response (P=0.049 and P<0.001, respectively), and predicted shorter PFS (P=0.002 and P<0.001, respectively) (*Online Supplementary Figure S3)*. The median pre-treatment and interim EBV DNA load were 880 (range 0-200,000) IU/mL and 141 (range 0-1,280,000) IU/mL in patients with final SD/PD response, respectively.

WGS/WES and targeted DNA-sequencing were performed on 128 patients. Gene mutations are summarized in Figure 3A. *JAK3* mutation was associated with inferior PFS and OS (Figure 3B, C), and *KMT2B* mutation predicted inferior PFS (*Online Supplementary Figure S4A, B*). Multivariate analysis incorporating baseline clinical characteristics and mutations found that *JAK3* mutation was an independent

Table 1. Main characteristics of the modified intention-to-treat population.

Characteristic	ESA N=125	MESA N=123
Gender, N (%)		
Female	41 (32.8)	29 (23.6)
Male	84 (67.2)	94 (76.4)
Age in years		
Median (range)	50 (14-70)	46 (15-70)
>60, N (%)	26 (20.8)	15 (12.2)
B symptoms, N (%)	52 (41.6)	43 (35.0)
Ann Arbor stage II, N (%)	48 (38.4)	45 (36.6)
Elevated lactate dehydrogenase, N (%)	47 (37.6)	48 (39.0)
Final response, N (%)		
CR	100 (80.0)	97 (78.9)
PR	11 (8.8)	9 (7.3)
SD	3 (2.4)	2 (1.6)
PD	7 (5.6)	11 (8.9)
Not evaluated	4 (3.2)	4 (3.3)
Median duration of response	NR	NR
Progression during the follow-up after first-line treatment, N (%)	14 (11.2)	12 (9.8)
Second cancers after first-line treatment, N (%)	1 (0.8)	1 (0.8)

CR: complete remission; ESA: etoposide, dexamethasone, and pegaspargase; MESA: methotrexate, etoposide, dexamethasone, and pegaspargase; N: number; NR: not reached; PD: progressive disease; PR: partial remission; SD: stable disease.

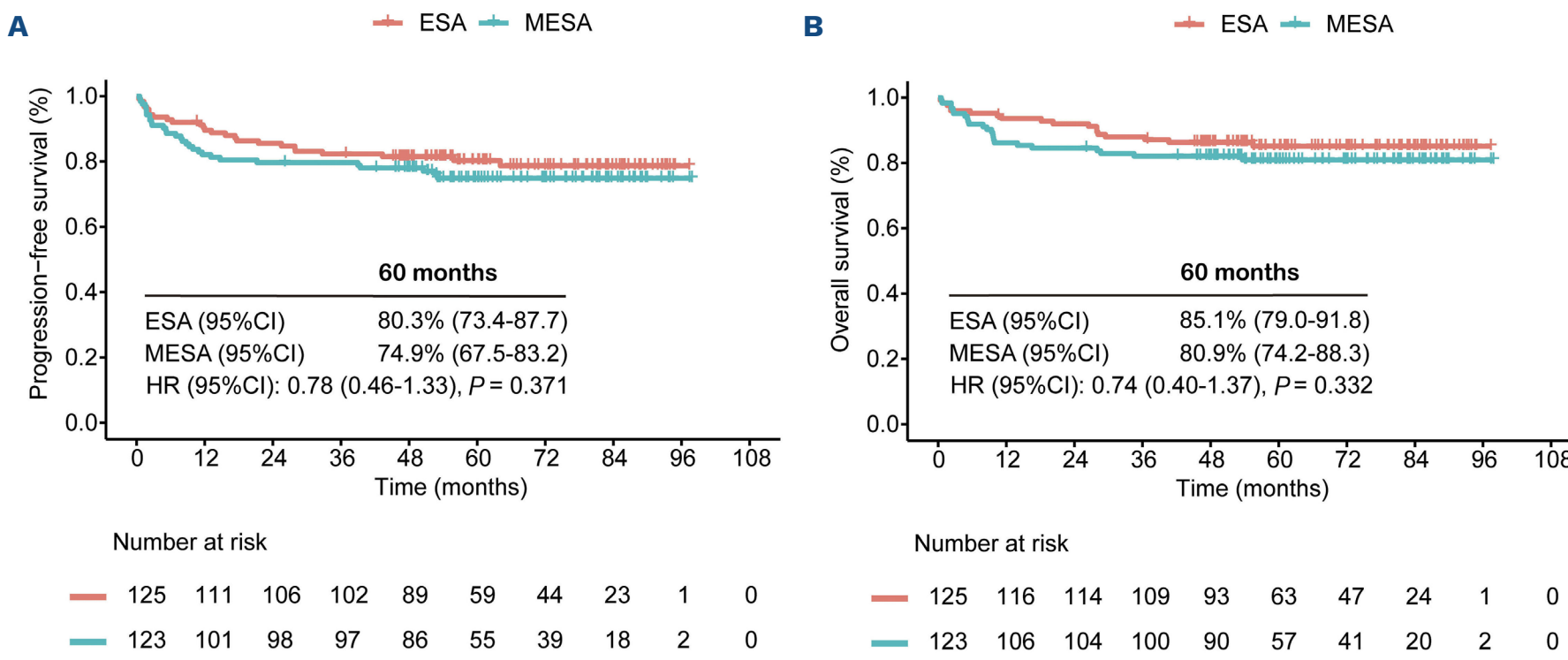

Figure 1. Long-term survival in modified intention-to-treat population. (A) Kaplan-Meier survival curve of progression-free survival (PFS) according to treatment arms. (B) Kaplan-Meier survival curve of overall survival according to treatment arms. (C) Forest plot of hazard ratios of PFS for patients with different subgroups, according to treatment arms. CA: Chinese Southwest Oncology Group and Asia Lymphoma Study Group ENKTL system; CI: confidence interval; EBV: Epstein-Barr virus; ECOG: Eastern Cooperative Oncology Group; ESA: etoposide, dexamethasone, and pegaspargase; HR: hazard ratio; IPI: International Prognostic Index; MESA: methotrexate, etoposide, dexamethasone, and pegaspargase; NRI: Nomogram-Revised Risk Index; PINK-E: Prognostic Index of Natural-Killer lymphoma-Epstein-Barr virus; y: years.

factor for PFS (HR, 8.59; 95% CI: 3.18-23.22, *P*<0.001) and OS (HR, 8.53; 95% CI: 2.95-24.68, *P*<0.001). However, it should be noted that *JAK3* and *KMT2B* mutations were presented in only 4.7% (N=6) and 5.5% (N=7) of the patients, respectively. Molecular subtypes were classified in 125 patients; the other 3 patients were unclassified without detective mutations or RNA-sequencing data. No significant difference was observed according to molecular subtypes for PFS and OS (*Online Supplementary Figure S4C, D*).

Biomarkers related to Epstein-Barr virus infection

To further investigate the potential mechanism of persistent EBV DNA infection during treatment, we analyzed the clinical characteristics, immunohistochemistry and RNA-sequencing. Female patients had higher proportions of interim EBV DNA positivity than male patients (*P*=0.020)

Table 2. Plasma Epstein-Barr virus DNA and interim response.

	ESA N=125	MESA N=123
Pre-treatment EBV DNA, N (%)		
Positive	57 (45.6)	58 (47.2)
Negative	68 (54.4)	65 (52.8)
EBV DNA after 2 cycles, N (%)		
Positive	10 (8.0)	16 (13.0)
Negative	101 (80.8)	90 (73.2)
NA	14 (11.2)	17 (13.8)
Post-treatment EBV DNA, N (%)		
Positive	7 (5.6)	13 (10.6)
Negative	92 (73.6)	79 (64.2)
NA	26 (20.8)	31 (25.2)
Interim response, N (%)		
CR	81 (64.8)	68 (55.3)
PR	30 (24.0)	36 (29.3)
SD	6 (4.8)	9 (7.3)
PD	4 (3.2)	6 (4.9)
Not evaluated	4 (3.2)	4 (3.2)

CR: complete remission; EBV: Epstein-Barr virus; ESA: etoposide, dexamethasone, and pegaspargase; MESA: methotrexate, etoposide, dexamethasone, and pegaspargase; N: number; NA: not available; PD: progressive disease; PR: partial remission; SD: stable disease.

Table 3. Univariate and multivariate analysis for progression-free survival and overall survival.

	Progression-free survival				Overall survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Pre-treatment								
Presence of B symptoms	1.20 (0.70-2.05)	0.504	1.11 (0.65-1.91)	0.701	2.19 (1.18-4.06)	0.013	1.98 (1.06-3.71)	0.032
Positive pre-treatment EBV DNA	1.63 (0.96-2.78)	0.073	1.60 (0.93-2.75)	0.087	1.96 (1.05-3.68)	0.035	1.74 (0.92-3.29)	0.089
Interim treatment								
Positive interim EBV DNA	3.88 (1.98-7.58)	< 0.001	2.45 (1.07-5.61)	0.033	4.72 (2.19-10.15)	< 0.001	3.11 (1.34-7.22)	0.008
Interim response PR *vs.* CR	0.99 (0.47-2.08)	0.984	1.24 (0.58-2.68)	0.576	1.52 (0.66-3.50)	0.330	2.11 (0.86-5.19)	0.105
Interim response SD *vs.* CR	3.18 (1.30-7.79)	0.011	3.13 (1.20-8.18)	0.020	4.07 (1.47-11.31)	0.007	3.70 (1.19-11.52)	0.024
Interim response PD *vs.* CR	-	-	-	-	25.11 (10.35-60.93)	< 0.001	17.04 (5.45-53.25)	< 0.001
Post-treatment								
Positive post-treatment EBV DNA	4.57 (2.00-10.40)	< 0.001	3.14 (1.17-8.47)	0.024	4.21 (1.48-11.95)	0.007	2.28 (0.69-7.51)	0.178
Final response PR/SD *vs.* CR	3.51 (1.57-7.83)	0.002	3.94 (1.34-11.62)	0.013	5.98 (2.47-14.45)	< 0.001	6.83 (2.12-21.96)	0.001
Final response PD *vs.* CR	-	-	-	-	30.67 (14.36-65.50)	< 0.001	15.61 (3.57-68.24)	< 0.001

CI: confidence interval; CR: complete remission; EBV: Epstein-Barr virus; HR: hazard ratio; PD: progressive disease; PR: partial remission; SD: stable disease.

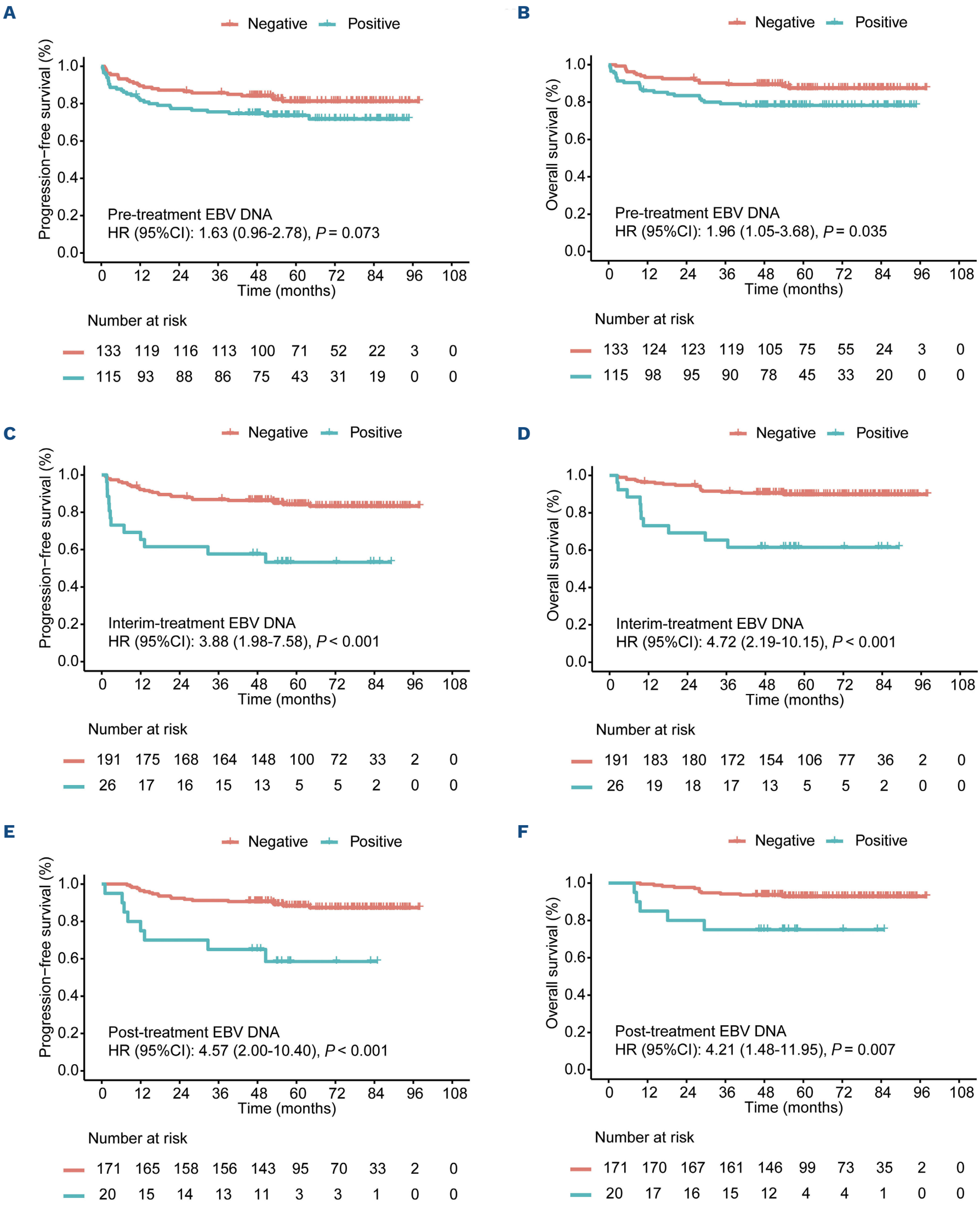

Figure 2. Progression-free survival and overall survival of Epstein-Barr virus DNA subgroups. Kaplan-Meier survival curve of progression-free survival (PFS) (A) and overall survival (OS) (B) according to pre-treatment Epstein-Barr virus (EBV) DNA. Kaplan-Meier survival curve of PFS (C) and OS (D) according to interim EBV DNA. Kaplan-Meier survival curve of PFS (E) and OS (F) according to post-treatment EBV DNA. CI: confidence interval; HR: hazard ratio.

(*Online Supplementary Table S3*). Pre-treatment MYC expression (P=0.046) and CD56 negativity (P=0.009) were significantly associated with interim EBV DNA positivity (Figure 4A, B). Next, RNA-sequencing was screened in 77 patients negative and 10 patients positive for interim EBV DNA. We compared differentially expressed genes and identified 176 up-regulated (e.g., *DUX4*, *DNMT3B*) and 206 down-regulated (e.g., *DMBT1*) genes in patients with interim EBV DNA

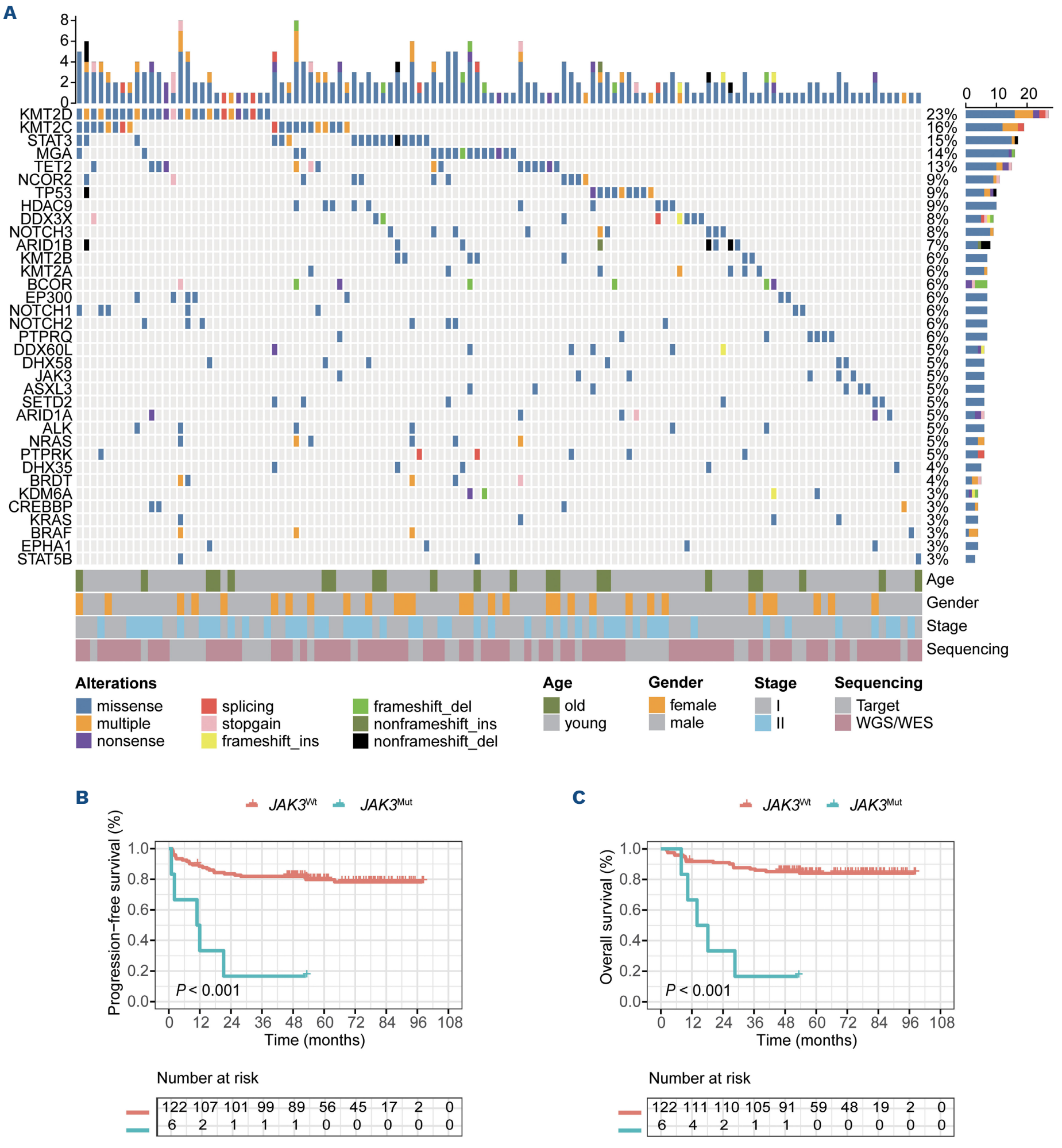

Figure 3. Genetic mutations. (A) Gene mutations landscape. Kaplan-Meier survival curve of progression-free survival (B) and overall survival (C) according to *JAK3* mutation. WES: whole exome sequencing; WGS: whole genome sequencing.

positivity (Figure 4C). Positive patients showed significant enrichment in neutrophil extracellular trap (NET) formation, ATP-dependent chromatin remodeling, viral carcinogenesis, metabolism (citrate cycle, 2-oxocarboxylic acid, carbon), and nucleocytoplasmic transport pathways (Figure 4D). The core-enrichment genes included *HDAC4, JAK3, RASA2*, and

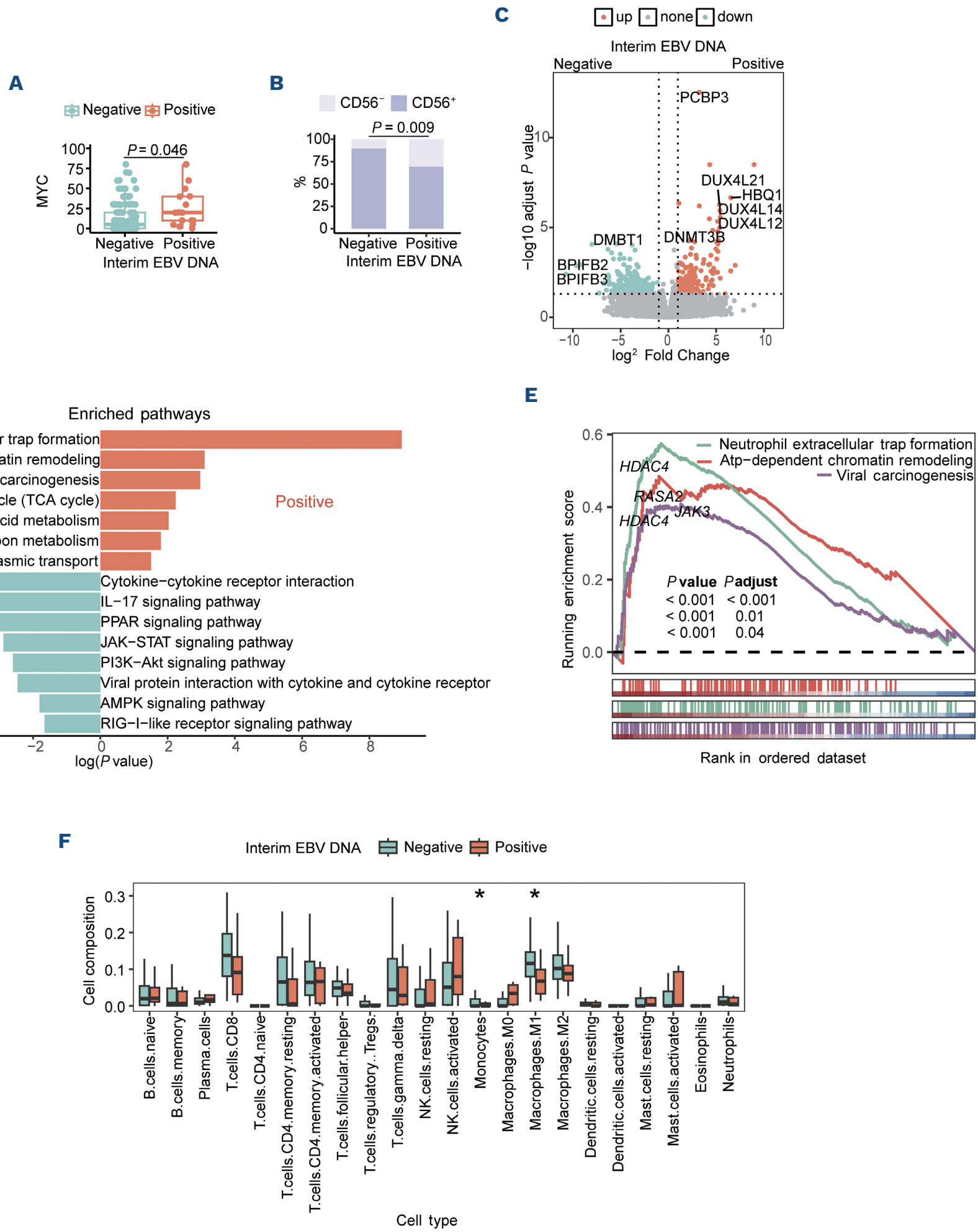

Figure 4. Transcriptomic signatures. (A) MYC and (B) CD56 expressions according to interim Epstein-Barr virus (EBV) DNA. (C) Differentially expressed genes according to interim EBV DNA. (D) Gene Set Enrichment Analysis revealed differential pathway alterations according to interim EBV DNA. (E) Up-regulated pathways in patients with interim EBV DNA positivity. (F) Infiltration of immune cells between positive and negative interim EBV DNA. NK: natural killer cells.

histones (Figure 4E). In contrast, immune-related pathways, such as cytokine-cytokine receptor interaction, IL-17 signaling, and viral protein interaction with cytokine and cytokine receptor were activated in negative patients (Figure 4D). Infiltration of immune cell subtypes were also assessed. Interim EBV DNA positivity was significantly associated with decreased infiltration of monocytes (*P*=0.017) and M1 macrophages (*P*=0.020), indicating an immunosuppressive microenvironment (Figure 4F).

Biomarkers related to disease progression

Biomarker analyses were also performed according to PFS. Patients with available RNA-seq data were categorized to the progression or remission group (N=22 *vs*. N=65). There were 100 up-regulated and 96 down-regulated genes in the progression group (*Online Supplementary Figure S5*). Pathways including oxidative phosphorylation, ribosome and cGMP-PKG signaling were enriched in patients with disease progression, while activated immune-related pathways were enriched in long-term remission patients, consistent with those of interim EBV DNA-negative patients. Decreased memory B cells and memory CD4 T cells were significantly associated with disease progression (*Online Supplementary Figure S5*).

According to time to progression, patients were further classified as early (PFS ≤12 months) and late (PFS >12 months) progression. Although no significant difference in baseline clinical characteristics was observed between the two groups (*Online Supplementary Table S4*), RNA-seq (N=12 *vs*. N=10) revealed 276 up-regulated genes in the early progression group, and 37 up-regulated genes in the late progression group (*Online Supplementary Figure S6*). Patients with early progression showed enrichment in pathways of NET formation, viral carcinogenesis, ATP-dependent chromatin remodeling, transcriptional dysregulation, and cholesterol metabolism, while patients with late progression showed activated cell adhesion molecules, signaling (Wnt, PPAR and calcium) and cytokine-cytokine receptor interaction pathways (*Online Supplementary Figure S6*).

Discussion

The updated 5-year analysis of the NHL-004 study provided substantial evidence that ESA with sandwiched radiotherapy has durable response and long-term survival in patients with newly diagnosed early-stage nasal NKTCL. With a median follow-up of 64 months, the 5-year PFS and OS rates were 80.3% and 85.1% in patients who received the ESA regimen with sandwiched radiotherapy. The biomarker analysis indicated that interim EBV DNA status and response were early independent predictors of PFS and OS during treatment. Interim EBV DNA positivity was strongly associated with up-regulated chromatin remodeling alterations and an immunosuppressive tumor microenvironment.

As compared to combined-modality therapy in early-stage NKTCL (*Online Supplementary Table S5*), for platinum-based chemotherapy with radiotherapy, the PFS and OS at five years in patients who received concurrent radiotherapy with DeVIC were 61% and 72%, respectively.[7] In the era of asparaginase, concurrent chemoradiotherapy followed by VIDL induced 5-year PFS and OS rates of 60% and 73%.[5] With GELOX regimens, the PFS and OS rates were 74% and 85% at five years.[6] The 5-year OS for the DDGP regimen was 74.3%.[9] Therefore, the anti-metabolic ESA regimen containing only intramuscular and oral agents, with a safe, outpatient design, maintains comparable response rate and long-term survival, and is a promising first-line treatment of early-stage NKTCL.

The prognostic value of pre- and post-treatment plasma EBV DNA has been proven in NKTCL. For patients receiving radiotherapy alone or with platinum- / asparaginase-containing regimens, detectable pre-treatment plasma EBV DNA correlates with tumor load and poor prognosis,[19-23] and is incorporated in the PINK-E scoring system.[16] Any detectable concentration was considered to be positive, and persistent EBV DNA was associated with worse PFS and OS.[22] In addition to pre-treatment EBV DNA positivity,[12] our long-term survival analysis further revealed that interim EBV DNA positivity was an independent predictor of PFS and OS, which should be considered as a useful predictor for early monitoring of poor outcomes in early-stage NKTCL. The EBV DNA status at interim represents a dynamic process that could better reflect individual sensitivity to pegaspargase-based treatment. Presence and high levels of EBV DNA at interim correlated with final treatment failure. Circulating EBV DNA could be related to high tumor burden and residual tumor cells.[20,24] In addition, persistent EBV infection may also contribute to genomic abnormalities and immune escape, further promoting oncogenesis, and affecting response and prognosis.[25,26]

Epigenetic modification, immune suppression, oncogenic signaling activation, metabolomic reprogramming and cell-cycle progression are hallmarks of NKTCL progression.[27,28] Pegaspargase and etoposide, used in the ESA regimen, directly target metabolic reprogramming and cell-cycle progression, respectively, but only partially overcame EBV infection in NKTCL. Status of interim EBV DNA revealed different genomic and transcriptomic characteristics. Over-expressed *DNMT3B* and *HDAC4* contribute to chromatin remodeling and epigenetic dysregulation.[29-32] Chromatin decondensation and microbial stimulation may trigger NET formation, which supports cancer progression and metastasis.[33,34] MYC and macrophage polarization create an immunosuppressive environment.[35-37] For example, up-regulated immune genes were identified as being related to persistent EBV infection. *DUX4*, expressed upon lytic replication of herpesviruses by viral proteins, suppresses MHC Class I to promote cancer immune evasion.[38,39] *RASA2* is revealed as a signaling checkpoint in human T cells, whose

ablation boosts antigen sensitivity and long-term function.[40] For patients with disease progression, the immunosuppressive microenvironment also plays an essential role.

These results provide physicians with evidence that treatment strategies could be adapted at interim based on interim response and interim EBV DNA status. This could be used in future clinical practice to further improve patient outcome. Novel agents could be effective for these patients, such as epigenetics-targeted drugs, immune checkpoint inhibitors, JAK inhibitor, and anti-EBV treatment.[41-45] According to the findings, another clinical trial (clinicaltrials.gov identifier 06069830) exploring interim positron emission tomography- and EBV DNA-directed therapy in localized nasal NKTCL is ongoing.

Since our biomarker analysis was post-hoc, a small number of patients were not available for interim plasma EBV DNA. Dynamic detection of plasma EBV DNA is warranted in future clinical trials and practices.

In conclusion, as an effective, low toxicity, non-intravenous regimen with an outpatient design, ESA with sandwiched radiotherapy achieved long-term durable response in patients with newly diagnosed early-stage NKTCL. Dynamic monitoring of plasma EBV DNA not only predicted disease progression, but also provided a clinical rationale for future mechanism-based therapy in NKTCL.

Disclosures

No conflicts of interest to disclose.

Contributions

Huijuan Zhong, Shu Cheng, Jie Xiong and WL Zhao investigated, collected, analyzed the data, and drafted the manuscript. WL Zhao, Saijuan Chen and PX conceived, designed and supervised the study. Jiayi Chen, RM, HY, Hongmei Yi, QS, Hao Zhang, YH, Guohui Cui, JW, Xi Zhang, BX, WQ, XH, MH, FY, XW, YS, Yuanhua Liu, XM, JH, FL, Chongyang Wu, Junmin Chen, LY, OB, Jingyan Xu, ZZ, LL, Xin Zhou, LH, YT, TN, DW, XJ, Chaofu Wang, BO, Gang Cai, BL, JL, ZL, RX, Luqun Wang, YJ, Yanyan Liu, Xiaoyun Zheng and Li Wang investigated and collected the data. All authors revised the manuscript and provided approval of the final version for submission.

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Data-sharing statement

The data reported in this article have been deposited in the NODE (http://www.biosino.org/node) platform (accession number OEP000498 and OEP003404).

References

1. Yang Y, Zhu Y, Cao JZ, et al. Risk-adapted therapy for early-stage extranodal nasal-type NK/T-cell lymphoma: analysis from a multicenter study. Blood. 2015;126(12):1424-1432.
2. Qi SN, Yang Y, Zhang YJ, et al. Risk-based, response-adapted therapy for early-stage extranodal nasal-type NK/T-cell lymphoma in the modern chemotherapy era: a China Lymphoma Collaborative Group study. Am J Hematol. 2020;95(9):1047-1056.
3. Yamaguchi M, Tobinai K, Oguchi M, et al. Phase I/II study of concurrent chemoradiotherapy for localized nasal natural killer/T-cell lymphoma: Japan Clinical Oncology Group Study JCOG0211. J Clin Oncol. 2009;27(33):5594-5600.
4. Wang L, Wang ZH, Chen XQ, et al. First-line combination of gemcitabine, oxaliplatin, and L-asparaginase (GELOX) followed by involved-field radiation therapy for patients with stage IE/IIE extranodal natural killer/T-cell lymphoma. Cancer. 2013;119(2):348-355.
5. Kim SJ, Yang DH, Kim JS, et al. Concurrent chemoradiotherapy followed by L-asparaginase-containing chemotherapy, VIDL, for localized nasal extranodal NK/T cell lymphoma: CISL08-01 phase II study. Ann Hematol. 2014;93(11):1895-1901.
6. Wang L, Wang ZH, Chen XQ, Wang KF, Huang HQ, Xia ZJ. First-line combination of GELOX followed by radiation therapy for patients with stage IE/IIE ENKTL: an updated analysis with long-term follow-up. Oncol Lett. 2015;10(2):1036-1040.
7. Yamaguchi M, Suzuki R, Oguchi M, et al. Treatments and outcomes of patients with extranodal natural killer/T-cell lymphoma diagnosed between 2000 and 2013: a cooperative study in Japan. J Clin Oncol. 2017;35(1):32-39.
8. Zhang Y, Ma S, Cai J, et al. Sequential P-GEMOX and radiotherapy for early-stage extranodal natural killer/T-cell lymphoma: a multicenter study. Am J Hematol. 2021;96(11):1481-1490.
9. Wang X, Zhang L, Liu X, et al. Efficacy and safety of a pegasparaginase-based chemotherapy regimen vs an L-asparaginase-based chemotherapy regimen for newly diagnosed advanced extranodal natural killer/T-cell lymphoma: a randomized clinical trial. JAMA Oncol. 2022;8(7):1035-1041.
10. Xiong J, Cui BW, Wang N, et al. Genomic and transcriptomic characterization of natural killer T cell lymphoma. Cancer Cell. 2020;37(3):403-419.e6.
11. Xiong J, Dai YT, Wang WF, et al. GPCR signaling contributes to immune characteristics of microenvironment and process of EBV-induced lymphomagenesis. Sci Bull (Beijing). 2023;68(21):2607-2619.
12. Zhong H, Cheng S, Zhang X, et al. Etoposide, dexamethasone, and pegaspargase with sandwiched radiotherapy in early-stage natural killer/T-cell lymphoma: a randomized phase III study. Innovation (Camb). 2023;4(3):100426.
13. Yahalom J, Illidge T, Specht L, et al. Modern radiation therapy for extranodal lymphomas: field and dose guidelines from the International Lymphoma Radiation Oncology Group. Int J Radiat Oncol Biol Phys. 2015;92(1):11-31.
14. Cheson BD, Fisher RI, Barrington SF, et al. Recommendations

for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. J Clin Oncol. 2014;32(27):3059-3068.
15. International Non-Hodgkin's Lymphoma Prognostic Factors P. A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med. 1993;329(14):987-994.
16. Kim SJ, Yoon DH, Jaccard A, et al. A prognostic index for natural killer cell lymphoma after non-anthracycline-based treatment: a multicentre, retrospective analysis. Lancet Oncol. 2016;17(3):389-400.
17. Hong H, Li Y, Lim ST, et al. A proposal for a new staging system for extranodal natural killer T-cell lymphoma: a multicenter study from China and Asia Lymphoma Study Group. Leukemia. 2020;34(8):2243-2248.
18. Chen SY, Yang Y, Qi SN, et al. Validation of nomogram-revised risk index and comparison with other models for extranodal nasal-type NK/T-cell lymphoma in the modern chemotherapy era: indication for prognostication and clinical decision-making. Leukemia. 2021;35(1):130-142.
19. Wang ZY, Liu QF, Wang H, et al. Clinical implications of plasma Epstein-Barr virus DNA in early-stage extranodal nasal-type NK/T-cell lymphoma patients receiving primary radiotherapy. Blood. 2012;120(10):2003-2010.
20. Kwong YL, Pang AW, Leung AY, Chim CS, Tse E. Quantification of circulating Epstein-Barr virus DNA in NK/T-cell lymphoma treated with the SMILE protocol: diagnostic and prognostic significance. Leukemia. 2014;28(4):865-870.
21. Yamaguchi M, Suzuki R, Kim SJ, et al. Early disease progression in patients with localized natural killer/T-cell lymphoma treated with concurrent chemoradiotherapy. Cancer Sci. 2018;109(6):2056-2062.
22. Li PF, Mao YZ, Bai B, et al. Persistent peripheral blood EBV-DNA positive with high expression of PD-L1 and upregulation of CD4 + CD25 + T cell ratio in early stage NK/T cell lymphoma patients may predict worse outcome. Ann Hematol. 2018;97(12):2381-2389.
23. Kim SJ, Choi JY, Hyun SH, et al. Risk stratification on the basis of Deauville score on PET-CT and the presence of Epstein-Barr virus DNA after completion of primary treatment for extranodal natural killer/T-cell lymphoma, nasal type: a multicentre, retrospective analysis. Lancet Haematol. 2015;2(2):e66-74.
24. Suzuki R, Yamaguchi M, Izutsu K, et al. Prospective measurement of Epstein-Barr virus-DNA in plasma and peripheral blood mononuclear cells of extranodal NK/T-cell lymphoma, nasal type. Blood. 2011;118(23):6018-6022.
25. Munz C. Latency and lytic replication in Epstein-Barr virus-associated oncogenesis. Nat Rev Microbiol. 2019;17(11):691-700.
26. Alsaadawe M, Radman BA, Long J, Alsaadawi M, Fang W, Lyu X. Epstein Barr virus: a cellular hijacker in cancer. Biochim Biophys Acta Rev Cancer. 2024;1879(6):189218.
27. Xiong J, Zhao WL. Advances in multiple omics of natural-killer/T cell lymphoma. J Hematol Oncol. 2018;11(1):134.
28. Tian XP, Cao Y, Cai J, et al. Novel target and treatment agents for natural killer/T-cell lymphoma. J Hematol Oncol. 2023;16(1):78.
29. Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature. 2011;476(7360):298-303.
30. Hoang NM, Rui L. DNA methyltransferases in hematological malignancies. J Genet Genomics. 2020;47(7):361-372.
31. Nebbioso A, Tambaro FP, Dell'Aversana C, Altucci L. Cancer epigenetics: moving forward. PLoS Genet. 2018;14(6):e1007362.
32. Cai JY, Xu TT, Wang Y, et al. Histone deacetylase HDAC4 promotes the proliferation and invasion of glioma cells. Int J Oncol. 2018;53(6):2758-2768.
33. Adrover JM, McDowell SAC, He XY, Quail DF, Egeblad M. NETworking with cancer: the bidirectional interplay between cancer and neutrophil extracellular traps. Cancer Cell. 2023;41(3):505-526.
34. Wang H, Kim SJ, Lei Y, et al. Neutrophil extracellular traps in homeostasis and disease. Signal Transduct Target Ther. 2024;9(1):235.
35. Jabs WJ, Wagner HJ, Maurmann S, Hennig H, Kreft B. Inhibition of macrophage inflammatory protein-1 alpha production by Epstein-Barr virus. Blood. 2002;99(5):1512-1516.
36. Fang Q, Stehr AM, Naschberger E, Knopf J, Herrmann M, Sturzl M. No NETs no TIME: crosstalk between neutrophil extracellular traps and the tumor immune microenvironment. Front Immunol. 2022;13:1075260.
37. Gao FY, Li XT, Xu K, Wang RT, Guan XX. c-MYC mediates the crosstalk between breast cancer cells and tumor microenvironment. Cell Commun Signal. 2023;21(1):28.
38. Chew GL, Campbell AE, De Neef E, et al. DUX4 suppresses MHC Class I to promote cancer immune evasion and resistance to checkpoint blockade. Dev Cell. 2019;50(5):658-671.e7.
39. Neugebauer E, Bastidas-Quintero AM, Weidl D, Full F. Pioneer factors in viral infection. Front Immunol. 2023;14:1286617.
40. Carnevale J, Shifrut E, Kale N, et al. RASA2 ablation in T cells boosts antigen sensitivity and long-term function. Nature. 2022;609(7925):174-182.
41. Shi Y, Dong M, Hong X, et al. Results from a multicenter, open-label, pivotal phase II study of chidamide in relapsed or refractory peripheral T-cell lymphoma. Ann Oncol. 2015;26(8):1766-1771.
42. Tang T, Martin P, Somasundaram N, et al. Phase I study of selinexor in combination with dexamethasone, ifosfamide, carboplatin, etoposide chemotherapy in patients with relapsed or refractory peripheral T-cell or natural-killer/T-cell lymphoma. Haematologica. 2021;106(12):3170-3175.
43. Tao R, Fan L, Song Y, et al. Sintilimab for relapsed/refractory extranodal NK/T cell lymphoma: a multicenter, single-arm, phase 2 trial (ORIENT-4). Signal Transduct Target Ther. 2021;6(1):365.
44. Song Y, Malpica L, Cai Q, et al. Golidocitinib, a selective JAK1 tyrosine-kinase inhibitor, in patients with refractory or relapsed peripheral T-cell lymphoma (JACKPOT8 Part B): a single-arm, multinational, phase 2 study. Lancet Oncol. 2024;25(1):117-125.
45. Tao R, Liu C, Zhang W, Zhu Y, Ma Y, Hao S. Selinexor with anti-PD-1 antibody as a potentially effective regimen for patients with natural killer/T-cell lymphoma failing prior L-asparaginase and PD-1 blockade. Oncologist. 2024;29(1):e90-e96.