REGULAR ARTICLE

blood advances

Genetic and immunohistochemical profiling of NK/T-cell lymphomas reveals prognostically relevant *BCOR*-MYC association

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Key Points

- Activating *STAT3* mutations are common in ENKTLs and associated with high CD30 expression.
- ENKTLs with deleterious *BCOR* mutations overexpress MYC, and this *BCOR*-MYC is associated with decreased overall survival.

Extranodal NK/T-cell lymphoma, nasal type (ENKTL) is an Epstein-Barr virus-positive, aggressive lymphoma with a heterogeneous cell of origin and variable clinical course. Several clinical prognostic indices have been proposed for ENKTL; however, there are few pathological biomarkers. This multi-institutional study sought to identify histologically assessable prognostic factors. We investigated mutation profiles by targeted next-generation sequencing (NGS) and immunohistochemical assessments of expression of MYC, Tyr705-phosphorylated (*p*-)STAT3, and CD30 in 71 ENKTL samples. The median age of the patients was 66 years (range, 6-100). The most frequent mutations were in *STAT3* (27%), *JAK3* (4%), *KMT2D* (19%), *TP53* (13%), *BCOR* (10%), and *DDX3X* (7%). Immunohistochemistry (IHC) revealed that ENKTLs with *STAT3* mutations exhibited higher expression of pSTAT3 and CD30. *BCOR* mutations were associated with increased MYC expression. Univariate analysis in the entire cohort showed that stage (II, III, or IV), *BCOR* mutations, *TP53* mutations, and high MYC expression (defined as ≥40% positive neoplastic cells) were associated with reduced overall survival (OS). Multivariate modeling identified stage (II, III, or IV) and high MYC expression as independent adverse prognostic factors. In a subgroup analysis of patients treated with anthracycline (AC)-free chemotherapy and/or radiotherapy (RT) with curative intent, *BCOR* but not high MYC expression was an independent adverse prognostic factor. In conclusion, activating *STAT3* mutations are common in ENKTLs and are associated with increased CD30 expression. MYC overexpression is, at least in part, associated with deleterious *BCOR* mutations, and this *BCOR*–MYC linkage may have prognostic significance, underscoring the potential utility of IHC for MYC in risk stratification of patients with ENKTL.

Introduction

Extranodal NK/T-cell lymphoma, nasal type (ENKTL) is an aggressive Epstein-Barr virus (EBV)-associated lymphoma of either cytotoxic T-cell or NK-cell origin.[1] ENKTL predominantly arises in the upper aero-digestive tract, such as the nasal cavity. Other less frequent primary sites include the skin, gastrointestinal tract, soft tissue, and testes. ENKTL is a rare disease accounting for approximately 10% of T/NK-cell

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The full-text version of this article contains a data supplement.

lymphomas[2]; however, its frequency varies geographically; it is relatively common in Asian or Pacific Islander, Hispanic White, and American Indian or Alaskan Native, and rarer in non-Hispanic White and Black populations.[3]

The clinical presentation of ENKTL is typically aggressive. Localized ENKTL is generally treated with concurrent chemoradiotherapy, such as radiotherapy (RT) with dexamethasone, etoposide, ifosfamide, and carboplatin (RT-DeVIC), with a 5-year overall survival (OS) rate of approximately 70%.[4,5] In contrast, the survival of patients with advanced ENKTL is poor, and despite intensive multiagent chemotherapy containing L-asparaginase, the 5-year OS rate is approximately 25%.[4,5] Nonetheless, clinical heterogeneity of ENKTL has been noted. For example, ENKTLs involving the non-upper aerodigestive tract region are more frequently disseminated, with a shorter 5-year OS than those involving the upper aerodigestive tract.[6] Given such clinical heterogeneity, there have been multiple clinical prognostic or predictive models proposed, incorporating age, serum lactate dehydrogenase (LDH) concentration, performance status, stage, and nonnasal disease.[5,7,8]

Reflecting its diverse clinical courses, ENKTL molecular heterogeneity has been recognized. As ENKTL, by definition, involves neoplastic proliferation of NK or T cells, T-cell receptor (TCR) gene rearrangement and protein expression of TCR are detected in the subset of ENKTLs derived from T cells.[9-11] Next-generation sequencing (NGS) profiling has identified commonly mutated genes, including *TP53*, *DDX3X*, *STAT3*, *JAK3*, *MGA*, and *BCOR*.[12-18] A recent genomic and transcriptomic study proposed molecular subclassifications into 3 subtypes: (1) TSIM, characterized by alterations in tumor suppressors such as *TP53* and immune modulators including JAK/STAT pathway genes and *PD-L1/2* amplification; (2) HEA, characterized by aberrant histone acetylation driven by mutations in *HDAC9*, *EP300*, and *ARID1A*; and (3) MB, enriched for *MYC*-associated aberrations such as *MGA* mutation and loss of heterozygosity (LOH) at the *BRDT* locus.[13] Interestingly, MB ENKTLs had significantly worse OS and progression-free survival (PFS) than those of the TSIM or HEA subtypes.

Despite these advances in understanding the clinical heterogeneity and molecular pathogenesis of ENKTL, there currently are few well-established pathological biomarkers.[19] Because ENKTL typically presents with extranodal lesions, biopsy specimens are often small, yielding limited amounts of DNA and RNA, so extensive molecular analysis is often challenging. Therefore, there remains a need for a histological prognostic biomarker that reflects molecular abnormalities. In this multi-institution study, we performed mutational and immunohistochemical characterization of 71 ENKTLs, identifying prognostically relevant *BCOR*–MYC axis.

Materials and methods

Case selection and data acquisition

This retrospective study was approved by the institutional review board of the University of Yamanashi Hospital (approval number 2195) and other related hospitals. It was performed with the Declaration of Helsinki. We retrieved ENKTL cases diagnosed at the University of Yamanashi, Aichi Medical University Hospital, Mayo Clinic, and Tokai University Hospital from 2000 to 2020, for a total of 71 cases. These included cases were nearly diagnosed consecutively, but a small number of cases were excluded because of insufficient specimens for additional pathological and molecular analyses. Cytologic features of 1 ENKTL involving the uterine cervix have been previously reported.[20] All ENKTL cases were histopathologically diagnosed on the basis of expression of T/NK-cell lineage markers, positivity for EBV-encoded small RNA, and at least focal expression of cytotoxic molecules such as granzyme B. No primary nodal EBV-positive T/NK-cell lymphomas were included. The following clinical information was retrieved from the medical records: age, sex, B symptoms, serum LDH concentration, stage, presence of nasal lesions, regional and distant lymph node involvement, NK/T-cell lymphoma prognostic index (NKPI),[8] prognostic index of NK-cell lymphoma (PINK),[7] and treatment. The median follow-up period was 12.5 months

Immunohistochemistry (IHC)

We measured the expression of 3 biomarkers, CD30, pSTAT3 (Tyr705-phosphorylated STAT3), and MYC, in ENKTLs (n = 71). Standard, indirect IHC was performed with antibodies specific for CD30 (clone 1G12, ready to use, Nichirei; Tokyo, Japan), pSTAT3 (clone D3A7, dilution 1:400; Cell Signaling Technology, Danvers, MA), and MYC (clone Y69, dilution 1:200, Abcam; Cambridge, UK). Membranous reactivity for CD30 and nuclear reactivity for pSTAT3 and MYC, at any intensity, were scored as positive on the single-cell level, then the sample was scored as the percentage of overall neoplastic cells in a sample with 10% increments. Expression was scored as high if positivity was ≥30% for CD30, ≥30% for pSTAT3, and ≥40% for MYC, as reported previously.[21,22]

NGS

For 67 ENKTL samples, we performed hybridization capture-based NGS, targeting 29 genes frequently mutated in T/NK-cell lymphomas: *ARID1A*, *ASXL3*, *BCOR*, *CDKN2A*, *DDX3X*, *DNMT3A*, *ECSIT*, *EP300*, *FAT4*, *HDAC9*, *IDH1*, *IDH2*, *JAK1*, *JAK2*, *JAK3*, *KMT2C*, *KMT2D*, *MGA*, *MSN*, *NRAS*, *STAT1*, *STAT3*, *STAT5A*, *STAT5B*, *STAT6*, *TET1*, *TET2*, *TET3*, and *TP53*. Briefly, DNA was extracted using the AllPrep DNA/RNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) and QIAcube (QIAGEN). DNA was sheared into 200- to 300-bp fragments with an M220 focused ultrasonicator (Covaris, Woburn, MA) and repaired with NEBNext FFPE DNA Repair Mix (M6630; New England Biolabs, Ipswich, MA). We prepared libraries using the xGen Prism DNA Library Prep Kit and enriched for full coding sequences of the 29 genes using xGen Predesigned Gene Capture Pools and the xGen Hybridization and Wash Kit (Integrated DNA Technologies, Coralville, IA). Sequencing was performed with an Illumina NovaSeq 6000 instrument. Reads were trimmed by fastp version 0.20 using default parameters.[23] We generated unmapped BAM files using FastqToSam (Picard Tools version 2.21.4, https://broadinstitute.github.io/picard). Reads were deduplicated with their start–stop positions, and unique molecular identifiers were created according to the manufacturer's protocol. The resulting BAM files mapped to hg19 were imported into CLC Genomics Workbench version 21 (https://digitalinsights.qiagen.com/) and locally realigned. Putative somatic variants were called using 5 filters: (1) read length ≥20; (2) sequencing coverage (depth) ≥50; (3) variant read count ≥5; (4) allele frequency ≥0.05; and (5) relative read direction filter with a significance of 0.01. Variants were annotated using Annovar.[24] SNPs, insertions, and deletions (indels) reported in the ExAC/gnomAD[25] and ToMMo[26] databases with allele frequencies >0.001 were excluded. We also required variants to have a frequency within the

Table 1. Clinicopathologic characteristics of patients with ENKTL

Characteristic	n = 71
Age, median (range), y	66 (6-100)
Sex, male/female, n (%)	41 (58)/30 (42)
B symptoms, yes/no, n (%)	21 (36)/37 (64)
LDH above the UNL, yes/no, n (%)	38 (64)/21 (36)
Stage, n (%)	
I	29 (49)
II	12 (20)
III	3 (5)
IV	15 (25)
Nasal lesion, yes/no, n (%)	45 (70)/19(30)
Regional LN involvement, yes/no, n (%)	20 (33)/41 (67)
Distant LN involvement, yes/no, n (%)	7 (11)/54 (89)
NKPI group, n (%)	
Group 1	10 (17)
Group 2	22 (38)
Group 3	10 (17)
Group 4	16 (28)
PINK risk, n (%)	
Low	21 (36)
Intermediate	27 (47)
High	10 (17)
Treatment, n (%)	
AC-free CTX and/or RT	45 (74)
Other CTX	8 (13)
No (including BSC)	8 (13)

AC, anthracycline; BSC, best supportive care; CTX, chemotherapy; LN, lymph node; RT, radiotherapy; UNL, upper normal limit.

ENKTL dataset of <30% for single-nucleotide variants and <5% for indels to exclude obvious alignment artifacts. Finally, variants meeting ≥1 of the following 3 criteria were scored as significant: (1) start loss, stop gain, or frameshift/nonframeshift indel; (2) designated as pathogenic/likely pathogenic in ClinVar[27]; (3) present ≥2 times in the COSMIC database v94 (https://cancer.sanger.ac.uk/cosmic). All reported variants were validated by manual inspection on the Integrative Genomics Viewer[28] and visualized by the cBioPortal OncoPrinter.[29,30] NGS is deposited in the Japanese Genotype–Phenotype Archive (accession number: JGAS000548).

Statistical analysis

The Fisher exact test was used to analyze ordinal or nominal categorical variables. The Wilcoxon rank sum test was used to analyze continuous numerical data. The log-rank test was used to compare OS, as determined using the Kaplan-Meier method. Univariate and multivariate Cox proportional hazards models were used to identify specific variables associated with OS. For the multivariate analysis, variables with a $P < .10$ in the univariate analyses were included. All statistical analyses were performed using JMP Pro 16.2.0 (SAS Institute, Cary, NC). Graphs were generated using GraphPad Prism 8 (GraphPad Software LLC, San Diego, CA).

Results

Clinicopathologic characteristics

Clinical data for the 71 patients with ENKTL are summarized in Table 1. Their median age was 66 years (range, 6-100), with a slight male predominance (58%). Nasal lesions were identified in 70% (45 of 64) of cases. Advanced stages (III or IV) were noted in 30% (18 of 59). Group 3/4 NKPI and high-risk PINK were observed in 45% (26 of 58) and 17% (10 of 58) of the patients, respectively. Primarily because of poor performance status, 13% (8 of 61) of the patients received no treatment. For patients with stage I or II disease and available treatment history, 71% (25 of 35) of the patients were treated with concurrent RT-DeVIC. Among patients with stage III or IV disease who received multiagent chemotherapy, 40% (6 of 15) were treated with dexamethasone, methotrexate, ifosfamide, *l*-asparaginase, and etoposide (SMILE). Over a median follow-up period of 12.5 months, the median OS was 85 months, and 5-year OS was 52% (supplemental Figure 1 in the data supplement).

IHC revealed that the median positivity (range) for pSTAT3, CD30, and MYC was 50% (range, 0%-100%), 20% (range, 0%-90%), and 30% (range, 0%-30%), respectively. High expression of pSTAT3, CD30, and MYC (≥30%, ≥30%, and ≥40%, respectively) was observed in 65% (46 of 71), 42% (30 of 71), and 48% (34 of 71) of ENKTLs, respectively.

Mutation profiling

Targeted NGS for 29 genes was performed for 67 out of 71 ENKTLs examined; mutation profiles are shown in Figure 1 and listed in supplemental Table 1. A total of 125 variants were identified in 79% (53 of 67) of ENKTLs, whereas the remaining 21% (14 of 67) had no mutations in the 29 genes. The most frequently mutated gene was *STAT3* in 27% of the patients (n = 18). Other JAK/STAT pathway genes were mutated at lower frequencies: *JAK3* (4%, n = 3), *STAT1* (4%, n = 3), *JAK1* (1.5%, n = 1), and *JAK2* (1.5%, n = 1). Consistent with earlier studies,[13,15,18,31] *TP53* mutations were relatively common, in 13% (n = 9) of cases. Recurrent mutations involved epigenetic modifier genes, including *KMT2D* (19%, n = 13), *TET2* (10%, n = 7), *DNMT3A* (9%, n = 6), *EP300* (9%, n = 6), *ARID1A* (7%, n = 5), *KMT2C* (7%, n = 5), and *HDAC9* (4%, n = 3). Other frequently mutated genes were *FAT4* (13%, n = 9), *BCOR* (10%, n = 7), *MGA* (7%, n = 5), and *DDX3X* (7%, n = 5). Activating *NRAS* missense mutations (p.G13D and p.Q61K) were found in 3% (n = 2) of ENKTLs.

Activating *STAT3* mutations are associated with high pSTAT3 and CD30 expression

Next, we investigated the clinicopathological significance of *STAT3* mutations in ENKTL, as it was the most frequently mutated gene in our ENKTL cohort. All *STAT3* mutations identified were missense mutations, with all but 2 (16 of 18) in the SH2 domain of the *STAT3* gene (Figure 2A). All *STAT3* mutations, including 2 substitutions in the non-SH2 domain (p.R152W and p.A703T), have been reported to be activating, gain-of-function mutations associated with T/NK-cell malignancies or familial lymphoproliferation and early onset solid-organ autoimmunity.[32,33] Consistent with their identification as activating mutations, ENKTLs with *STAT3* mutations exhibited significantly higher pSTAT3 expression by IHC than those without (median positivity 80% vs 40%; Wilcoxon test

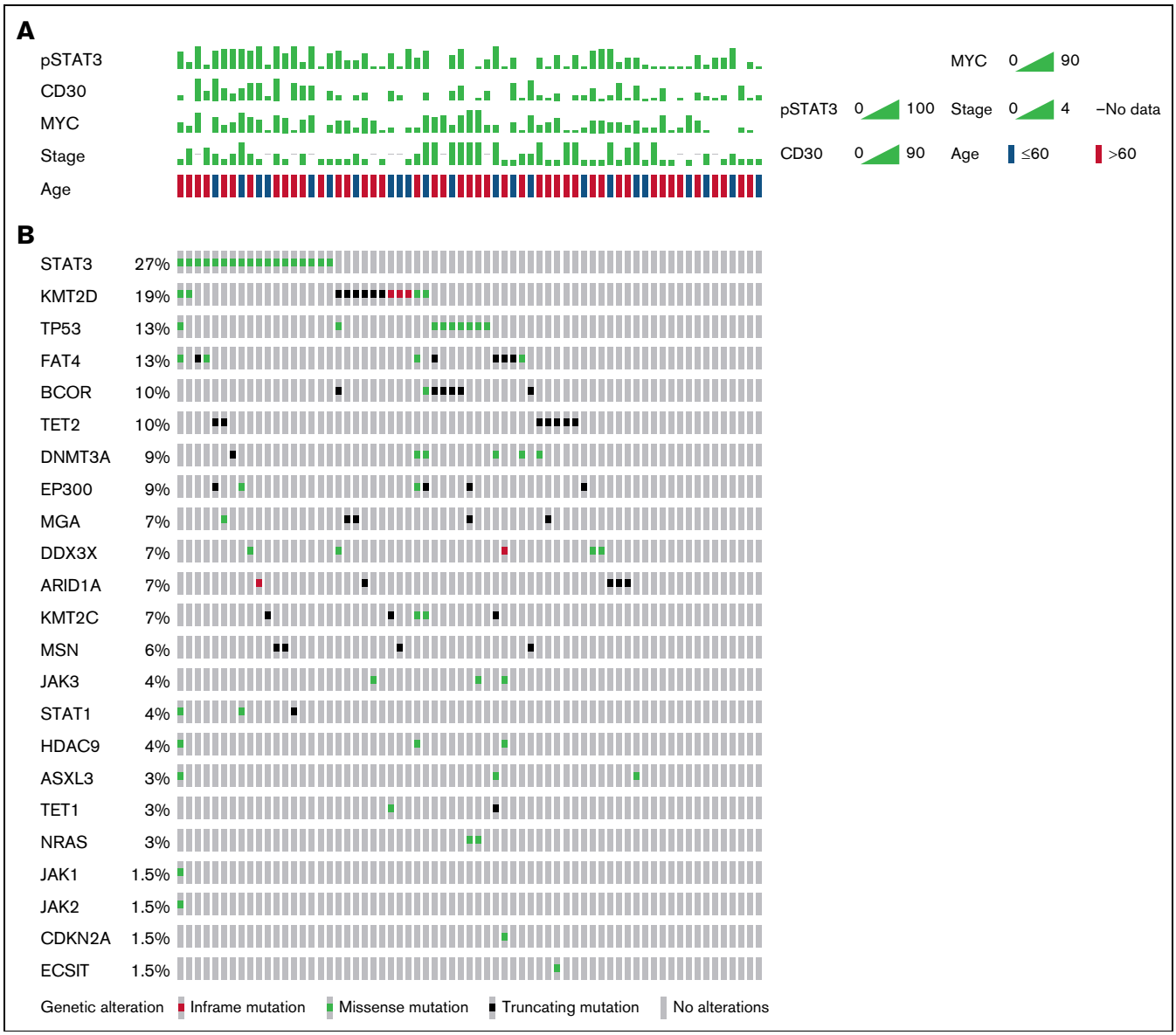

Figure 1. ENKTL mutation profiles from targeted NGS. (A) Clinical and IHC characteristics. (B) Mutation profiles of 29 examined genes.

$P < .001$) (Figure 2B,E). Furthermore, CD30 was expressed at higher concentrations in ENKTLs with *STAT3* mutations than in those without (median positivity, 50% vs 20%; Wilcoxon test $P = .002$) (Figure 2C,E). In contrast, there was no significant association between *STAT3* mutation status and MYC expression (median positivity, 45% vs 30%; Wilcoxon test $P = .229$) (Figure 2D). Survival analysis showed no significant difference in OS for patients with or without *STAT3* mutations (log-rank test $P = .628$) (Figure 2F).

We have identified a few mutations in JAK/STAT pathway genes other than *STAT3*, including *JAK3* (4%, n = 3), *STAT1* (4%, n = 3), *JAK1* (1.5%, n = 1), and *JAK2* (1.5%, n = 1). Two *JAK3* variants, p.A573V (n = 1) and p.M511I (n = 1), were activating and identified in ENKTLs with wild-type *STAT3*. The functionality of the other variants was of uncertain significance and mostly overlapped with *STAT3* mutations (Figure 1B). Of note, after inclusion of non-*STAT3* mutations, ENKTLs with a JAK/STAT gene mutation exhibited significantly higher expression of pSTAT3 (median positivity, 80% vs 40%; Wilcoxon test $P = .013$) and CD30 (median positivity, 40% vs 20%; Wilcoxon test $P = .037$) (supplemental Figure 2).

BCOR mutations are associated with high MYC expression and decreased OS

We focused on mutations in the gene encoding the BCL6 corepressor BCOR and their clinicopathological significance in ENKTLs. *BCOR* mutations were identified in 10% (7 of 67) of ENKTLs (Figure 1B); all but 1 missense mutation (p.V594I) were deleterious frameshift or nonsense mutations (Figure 3A). All ENKTLs with *BCOR* mutations tested negative for *STAT3* mutations. In contrast, there was a tendency for cooccurrence between *BCOR* and *TP53* mutations; 71% (5 of 7) of *BCOR*-mutated ENKTLs had *TP53* mutations, and 55% (5 of 9) of *TP53*-mutated

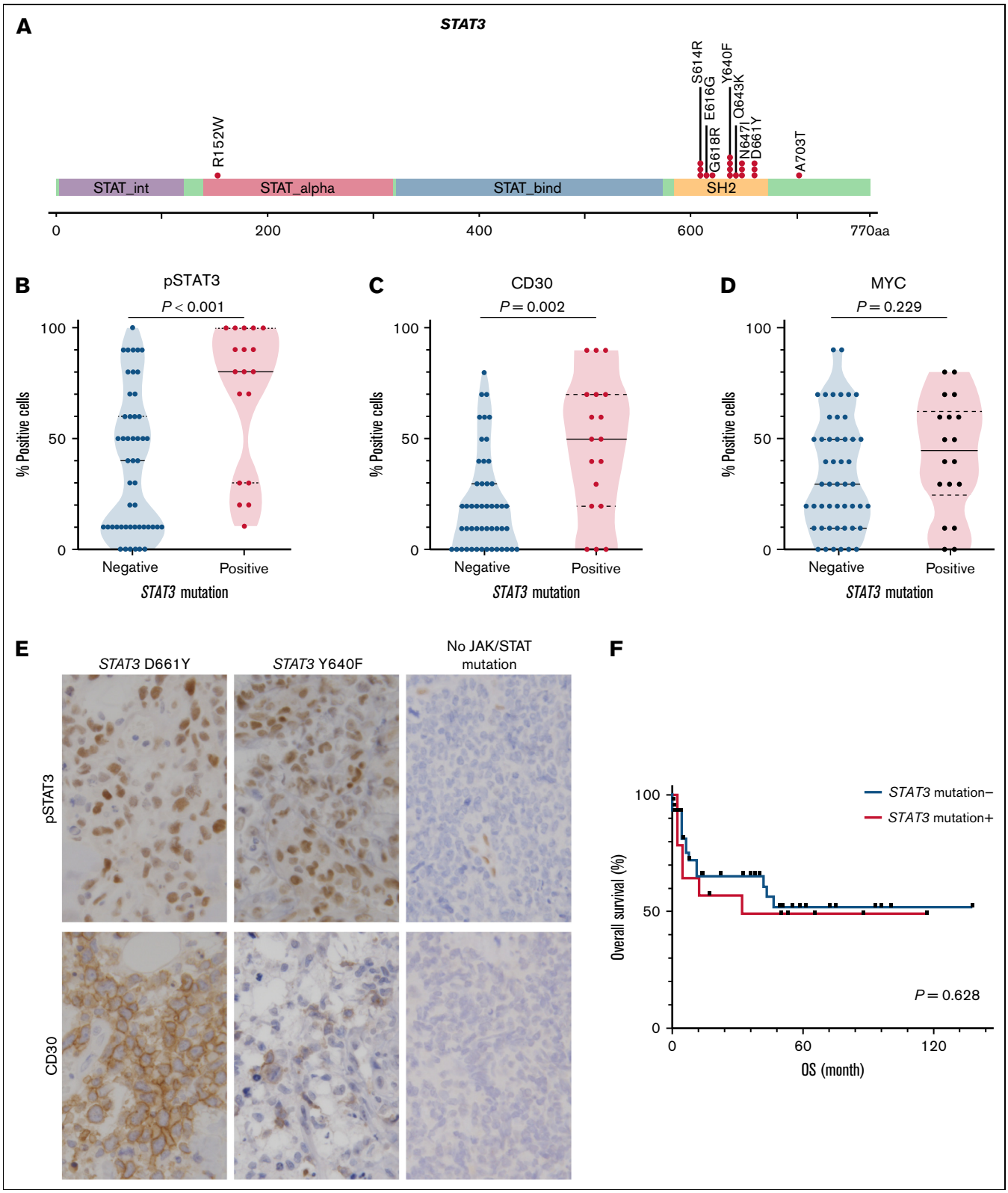

Figure 2. *STAT3* mutations in ENKTLs. (A) Mutation distribution. (B-D) Semiquantitative measurements of pSTAT3, CD30, and MYC expression as a function of *STAT3* mutation status. (E) Representative IHC images for pSTAT3 and CD30 by *STAT3* mutation status. Original magnification ×400. (F) Kaplan-Meier plot of OS as a function of *STAT3* mutation status.

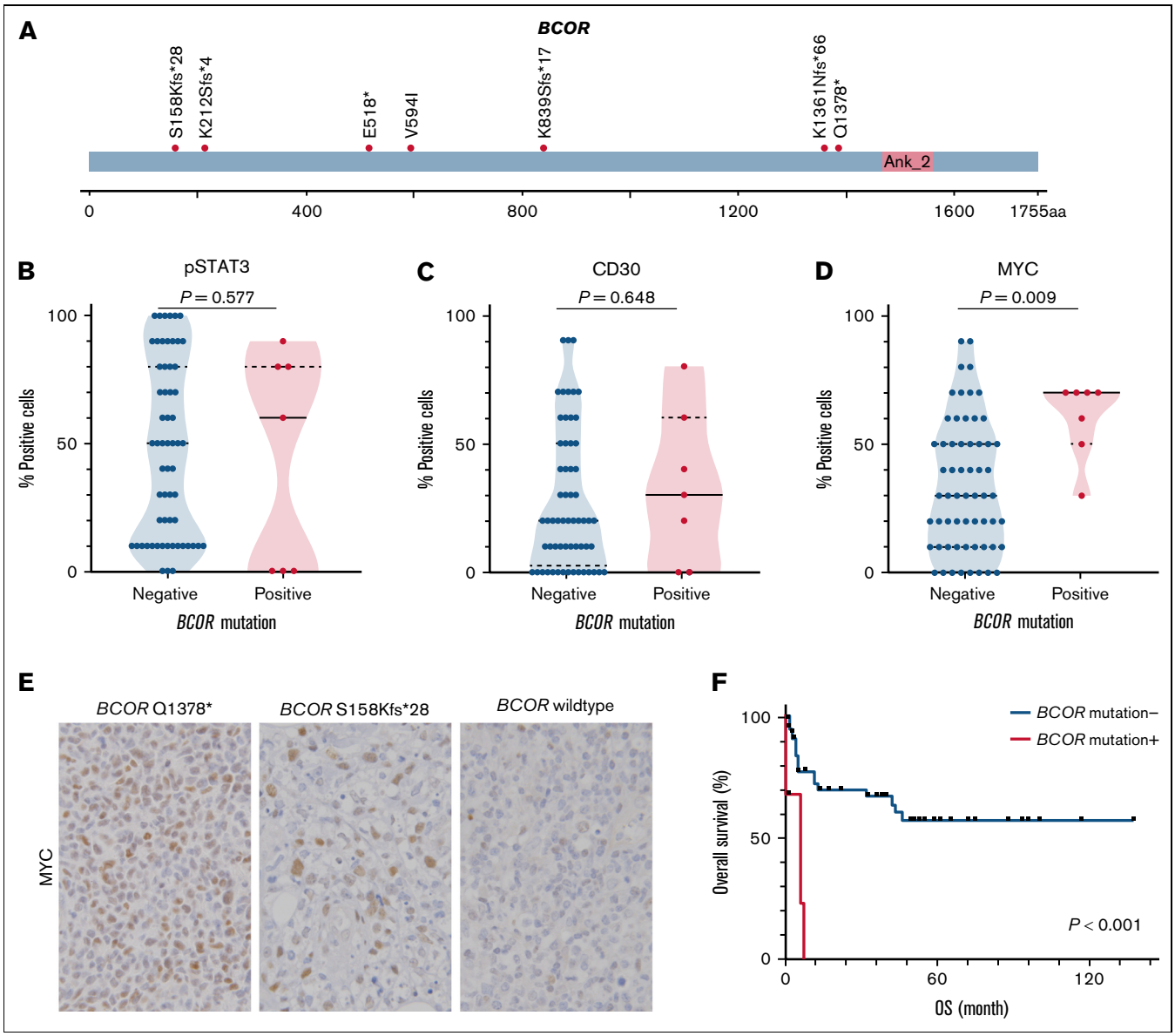

Figure 3. *BCOR* mutations in ENKTLs. (A) Mutation distribution. (B-D) Semiquantitative measurements of pSTAT3, CD30, and MYC expression as a function of *BCOR* mutation status. (E) Representative IHC images for MYC by *BCOR* mutation status. Original magnification ×400. (F) Kaplan-Meier plot of OS as a function of *BCOR* mutation status.

cases had *BCOR* mutations ($P < .001$; q-value = 0.062). While *BCOR* mutation status was not significantly associated with pSTAT3 and CD30 expression levels by IHC (Figure 3B-C), ENKTLs with *BCOR* mutations showed significantly higher MYC expression than those without *BCOR* mutations (median positivity 70% vs 30%; Wilcoxon test $P = .009$) (Figure 3D-E). Of note, patients with *BCOR* mutations (n = 7) had a significantly shorter survival than those without *BCOR* mutations (n = 52) (median OS, 5.9 months vs undefined; log-rank test $P < .001$) (Figure 3F).

High MYC expression and *BCOR* mutation are adverse prognostic factors in ENKTL

We examined OS with respect to known prognostic factors, genetic alterations, and IHC markers (Figure 4). Patients with stage II/III/IV disease (n = 30) had a significantly shorter OS than those with stage I disease (n = 29) (median OS, 11.4 months vs undefined; log-rank test, $P = .008$) (Figure 4A). In addition to the above-mentioned *BCOR* mutations, ENKTLs with *TP53* mutations (n = 8) had significantly decreased OS than those without (n = 51) (median OS, 5.9 months vs undefined; log-rank test $P = .002$) (Figure 4B). Among the 3 IHC markers examined, ENKTLs with high MYC expression defined by positivity ≥40% (n = 31) showed inferior OS compared with those with low MYC expression (median OS 32.1 months vs undefined; log-rank test $P = .020$) (Figure 4E). No statistically significant association was observed between OS and pSTAT3 ($P = .121$) or CD30 ($P = .195$) expression (Figure 4C-D).

The univariate and multivariate analyses in the entire cohort are summarized in Table 2. In the univariate analyses, we identified that

Table 2. Univariate and multivariate analyses of factors associated with OS of overall patients with ENKTL

Variable	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
Age > 60 y						
No	1	–	–	–	–	–
Yes	0.70	0.32-1.57	.392	–	–	–
B symptoms						
No	1	–	–	–	–	–
Yes	1.29	0.55-2.98	.558	–	–	–
Stage						
I	1	–	–	1	–	–
II, III, or IV	3.07	1.30-7.26	.011	3.07	1.09-8.67	.034
Serum LDH > ULN						
No	1	–	–	–	–	–
Yes	2.00	0.79-5.11	.146	–	–	–
Nasal lesion						
Present	1	–	–	–	–	–
Absent	0.93	0.40-2.16	.863	–	–	–
Regional LN involvement						
No	1	–	–	–	–	–
Yes	1.24	0.54-2.83	.605	–	–	–
Distant LN involvement						
No	1	–	–	–	–	–
Yes	1.04	0.31-3.50	.952	–	–	–
Treatment						
AC–free CTX and/or RT	0.29	0.08-1.01	.051	0.22	0.06-0.88	.032
Other CTX	0.47	0.10-2.14	.331	0.45	0.07-2.97	.404
No (including BSC)	1	–	–	1	–	–
***STAT3* mutation**						
Negative	1	–	–	–	–	–
Positive	1.25	0.51-3.06	.630	–	–	–
***BCOR* mutation**						
Negative	1	–	–	1	–	–
Positive	6.66	2.25-19.69	<.001	5.11	0.56-46.97	.149
***TP53* mutation**						
Negative	1	–	–	1	–	–
Positive	4.06	1.55-10.59	.004	0.89	0.13-6.17	.902
pSTAT3 ≥ 30%						
No	1	–	–	–	–	–
Yes	2.04	0.81-5.11	.129	–	–	–
CD30 ≥ 30%						
No	1	–	–	–	–	–
Yes	1.67	0.76-3.67	.200	–	–	–
MYC ≥ 40%						
No	1	–	–	1	–	–
Yes	2.62	1.13-6.09	.025	3.92	1.28-12.02	.017

BSC, best supportive care; LN, lymph node; RT, radiotherapy; ULN, upper normal limit.

stage II, III, or IV disease (for OS: hazard ratio [HR], 3.07; 95% confidence interval [CI], 1.30-7.26; $P = .011$), *BCOR* mutation (HR, 6.66; 95% CI, 2.25-19.69; $P < .001$), *TP53* mutation (HR, 4.06; 95% CI, 1.55-10.59; $P = .004$), and high MYC expression (HR, 2.62; 95% CI, 1.13-6.09; $P = .025$) were associated with an increased risk of death (Table 2). In a multivariate analysis, we found that stage II, III, or IV disease (HR, 3.07; 95% CI, 1.09-8.67; $P = .034$) and high MYC expression (HR, 3.92; 95% CI, 1.28-12.02; $P = .017$) were independently associated with decreased survival (Table 2). In contrast, anthracycline (AC)-free chemotherapy and/or RT were associated with decreased risk of death compared with no treatment/best supportive care (HR, 0.22; 95% CI, 0.06-0.88; $P = .032$).

We then performed a subgroup analysis on patients treated with AC-free chemotherapy and/or RT with curative intent (Table 3). Careful interpretation of the results is needed because of the limited number of patients in the subgroup (n = 45). In a univariate analysis, stage II, III, or IV disease (HR, 2.92; 95% CI, 1.09-7.81; $P = .033$) and *BCOR* mutation (HR, 9.71; 95% CI, 2.57-36.72; $P < .001$) were associated with increased risk of death (Table 3). In the multivariate analysis, only *BCOR* mutations remained significant (HR, 6.41; 95% CI, 1.33-30.86; $P = .021$).

Discussion

In the present study, we investigated the mutational profile and protein expression of 71 ENKTLs using targeted NGS and IHC, revealing multiple genotype–immunophenotype associations with prognostic impacts. We confirmed that mutations that activate the JAK/STAT pathway are common in ENKTL. IHC for pSTAT3 revealed that 65% of the ENKTLs had JAK/STAT pathway activation, partly explained by activating mutations in JAK/STAT pathway genes, including *STAT3*, consistent with earlier reports on T/NK-cell malignancies.[31,34,35] The identification of frequent JAK/STAT mutations and active signaling offers insight into therapeutic approaches. A recent phase 2 study demonstrated that the JAK1/2 inhibitor ruxolitinib is clinically efficacious across multiple T-cell lymphoma subtypes, particularly for T-cell lymphomas with JAK/STAT mutations or active signaling defined by pSTAT3 IHC positivity ≥30%,[22] although this study enrolled no patients with ENKTL. Given the high frequency of JAK/STAT activation in ENKTLs and the promising efficacy of ruxolitinib in non-ENKTL T-cell lymphomas, although the controversial risk of secondary lymphoproliferative disorders should be carefully considered,[36-38] our data suggest that at least a subset of patients with ENKTL would benefit from this precise therapeutic approach to JAK/STAT inhibition. pSTAT3 was also positive in a considerable number of ENKTLs without JAK/STAT gene mutations, suggesting that there are additional underlying molecular mechanisms leading to pathway activation. Because our targeted NGS panel did not include genes encoding JAK/STAT pathway suppressors, such as *SOCS1* and *SOCS3*, loss-of-function mutations in them would provide a potential explanation,[39,40] although a previous whole-exome sequencing study on ENKTL reported no *SOCS* mutations.[13]

ENKTLs frequently exhibit increased CD30 expression[41-43]; using a cutoff of 30%, in our cohort, 42% were $CD30^+$. There was no significant difference in OS between $CD30^+$ and $CD30^-$ ENKTLs.

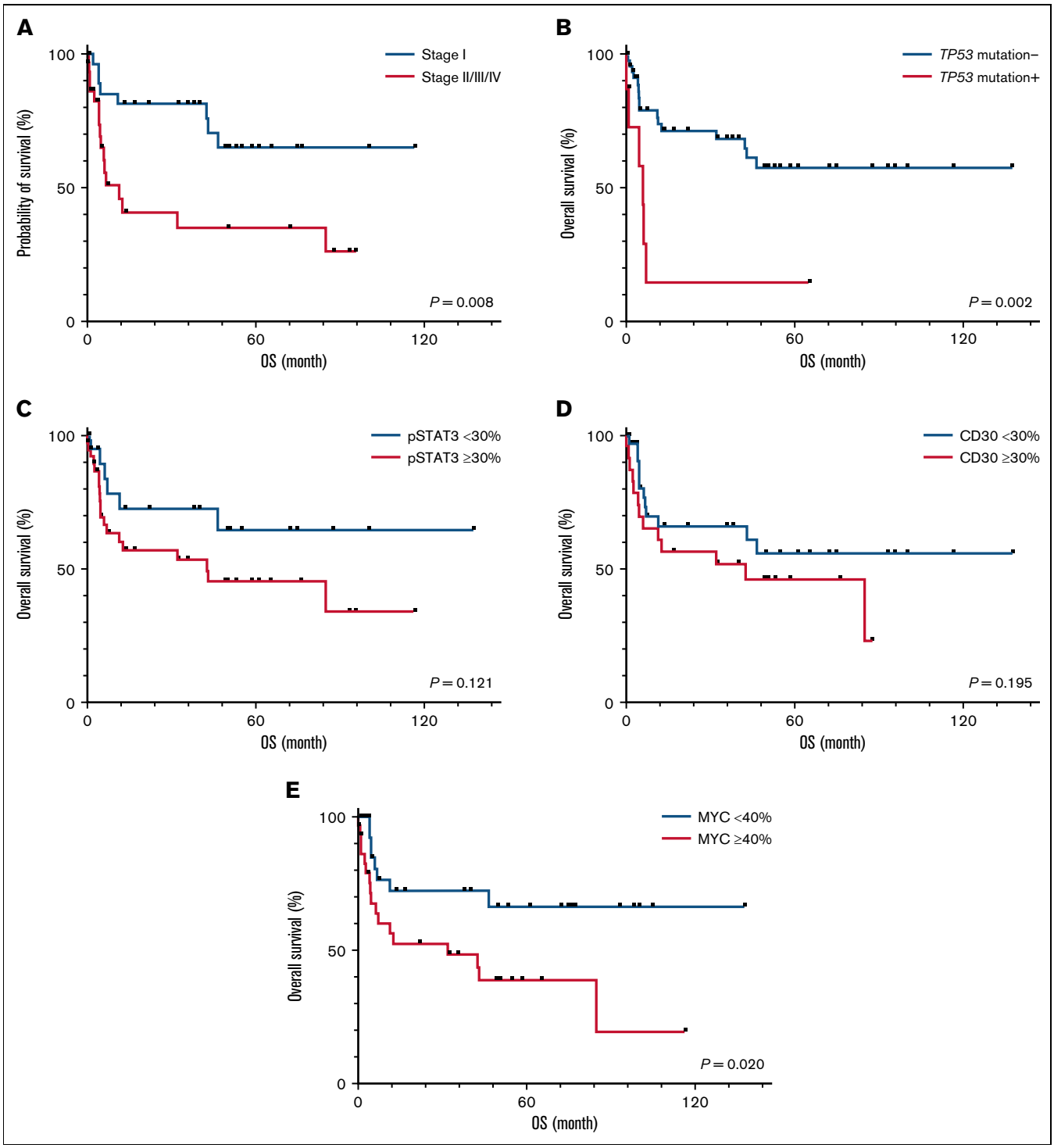

Figure 4. OS of patients with ENKTL. Kaplan-Meier plots of OS as a function of (A) stage, (B) *TP53* mutation, (C) pSTAT3, (D) CD30, and (E) MYC expression.

The prognostic impact of CD30 expression in ENKTL is controversial; one explanation could be the different cutoffs of CD30 IHC positivity used.[42] A systematic review and meta-analysis of 10 retrospective cohort studies reported that CD30 expression is associated with better OS in patients with ENKTL.[44] To our knowledge, ours is the first study to demonstrate an association between *STAT3* mutation and CD30 expression in ENKTL. High CD30 expression correlated with *STAT3* mutation, implying underlying molecular interactions between the activated JAK/STAT pathway and the *TNFRSF8* gene that encodes CD30. Supporting this hypothesis, a study using CRISPR library screening found that *TNFRSF8* is a transcriptional target of STAT3 and that its

Table 3. Univariate and multivariate analyses of factors associated with OS of patients with ENKTL treated with curative intent

	Univariate			Multivariate		
Variable	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age > 60 y						
No	1	–	–	–	–	–
Yes	0.45	0.18-1.16	.099	0.46	0.16-1.32	.149
B symptoms						
No	1	–	–	–	–	–
Yes	1.20	0.43-3.3	.729	–	–	–
Stage						
I	1	–	–	1	–	–
II, III, or IV	2.92	1.09-7.81	.033	1.77	0.55-5.73	.337
Serum LDH > ULN						
No	1	–	–	–	–	–
Yes	2.09	0.67-6.53	.204	–	–	–
Regional LN involvement						
No	1	–	–	–	–	–
Yes	1.33	0.5-3.59	.567	–	–	–
Nasal lesion						
Present	1	–	–	–	–	–
Absent	0.46	0.18-1.20	.112	–	–	–
Distant LN involvement						
No	1	–	–	–	–	–
Yes	1.51	0.34-6.65	.584	–	–	–
***STAT3* mutation**						
Negative	1	–	–	–	–	–
Positive	0.75	0.24-2.33	.617	–	–	–
***BCOR* mutation**						
Negative	1	–	–	1	–	–
Positive	9.71	2.57-36.72	<.001	6.41	1.33-30.86	.021
***TP53* mutation**						
Negative	1	–	–	–	–	–
Positive	2.69	0.75-9.62	.128	–	–	–
pSTAT3 ≥ 30%						
No	1	–	–	–	–	–
Yes	2.05	0.67-6.26	.206	–	–	–
CD30 ≥ 30%						
No	1	–	–	–	–	–
Yes	1.64	0.65-4.16	.299	–	–	–
MYC ≥ 40%						
No	1	–	–	–	–	–
Yes	1.96	0.73-5.26	.179	–	–	–

BSC, best supportive care; CTX, chemotherapy; LN, lymph node; RT, radiotherapy; UNL, upper normal limit.

transcription is significantly downregulated in STAT3-depleted anaplastic large cell lymphoma cell lines.[45] The CD30-directed antibody–drug conjugate brentuximab vedotin (BV) has been introduced for the treatment of peripheral T-cell lymphomas[46] and classic Hodgkin lymphomas[47] expressing CD30, although the proportion of $CD30^+$ tumor cells is not necessarily correlated with BV efficacy. However, there have been a limited number of studies on the efficacy of BV in ENKTLs, with varying overall response rates.[48-51] Therefore, it is pivotal to establish a predictive biomarker to identify patients with ENKTL who can respond best to BV. Collectively, our data suggest that CD30-directed therapy could be a therapeutic option for ENKTL, especially those harboring activating *STAT3* mutations.

We demonstrated that MYC overexpression is an adverse prognostic factor for patients with ENKTL. A limited number of reports have suggested the prognostic significance of high MYC expression. Huang and colleagues demonstrated that MYC expression in 53 ENKTLs, defining positivity as ≥5%, is associated with unfavorable OS and decreased PFS.[52] Wang and colleagues performed IHC for MYC in 53 ENKTLs, defining positivity as ≥20%, demonstrating an association with decreased PFS and OS.[53] A study by Chen and colleagues of 54 patients with ENKTL found that OS was significantly shorter in the MYC^+ group (defined as positivity ≥40%).[54] Although they used different cutoff values, all showed high MYC expression to be an independent prognostic factor by multivariate analyses. Our finding further confirms the utility of MYC IHC for risk stratification. Because IHC for MYC is widely used for the risk stratification of B-cell malignancies, especially diffuse large B-cell lymphoma,[55] the addition of MYC to a diagnostic IHC panel for ENKTL would be simple to implement and of significant prognostic value. Taken together, although the cutoff value needs to be validated, MYC overexpression determined by IHC is an adverse prognostic factor useful for stratifying the survival of patients with ENKTL. The precise molecular mechanism(s) underlying this association is currently unknown. An in vivo study showed that MYC overexpression upregulates transcription of the long noncoding RNA SNHG12, decreasing sensitivity to cisplatin[56]; however, further studies are required to elucidate the functional consequences of MYC overexpression in ENKTL.

The molecular mechanism underlying MYC overexpression in ENKTL is interesting since, unlike B-cell lymphomas, gene rearrangements involving MYC are absent or very rare, although increased *MYC* copy number is present in a subset of ENKTLs.[54] In the present study, ENKTLs with high MYC expression were enriched in those with mutations in the *BCOR* gene, encoding the BCL6 corepressor BCOR, which have been reported in T-cell– and NK-cell–derived malignancies with varying frequencies.[12,13,15-18] In ENKTL, the frequency of *BCOR* mutations varies from 0% to 32%.[12,13,15-18] Consistent with the observed association between high MYC expression and *BCOR* mutation in the current ENKTL series, an earlier study demonstrated that *BCOR* represses *MYC* transcription in T cells.[57] Mice missing *Bcor* exon 4, thus lacking the BCL6-binding domain of BCOR, showed a strong propensity to develop acute T-cell lymphoblastic leukemia (T-ALL); interestingly, *Myc* was highly upregulated in the T-ALL induced in the *Bcor*-deleted mice. Therefore, human ENKTLs with deleterious *BCOR* mutations likely represent an aggressive subgroup of ENKTL, which is presumably driven by aberrant MYC activation by abrogation of *BCOR*. In contrast, Xiong and colleagues identified a clinically aggressive ENKTL MB subtype, characterized by tumor suppressor gene *MGA* mutations, 1p22.1/*BRDT* LOH, and overexpression of MYC.[13] Like *BCOR*, *MGA* is a tumor suppressor that inhibits MYC-dependent tumor progression.[58] It is of interest whether the MB subtype reported by Xiong and colleagues overlaps with the

MYC-high ENKTLs in our series. We observed no significant association between *MGA* mutations (identified only in 7% of cases) and MYC expression (data not shown), although we did not test for 1p22.1/*BRDT* LOH. Another potential mechanism leading to high MYC expression is the activation of the JAK/STAT pathway: *MYC* is a downstream target of STAT3, and Song and colleagues reported that Ba/F3 cells forced to express mutant *STAT3* show higher mRNA expression levels of MYC.[59] In our ENKTL samples, however, there was no significant association between *STAT3* or JAK/STAT pathway gene mutations and MYC protein expression levels by IHC (Figure 2D; supplemental Figure 2). Collectively, there seem to be multiple molecular alterations capable of driving MYC overexpression and ENKTL aggressiveness, including *BCOR* and *MGA* mutations.

The present study has several limitations. ENKTL is uncommon, even in East Asian populations. Our multi-institutional study investigated the OS of 63 patients with ENKTLs incorporating clinicopathological, IHC, and genetic characteristics; however, the relatively small number of patients included was the primary limitation of our study. Another potential limitation is treatment heterogeneity. Because our case series dated from 2000, not all patients with advanced-stage ENKTL underwent current standard AC-free multiagent chemotherapies, such as SMILE.[60] MYC overexpression was an independent adverse prognostic factor in the entire cohort; however, it was not significant in the subgroup treated with AC-free chemotherapy and/or RT for curative intent. Therefore, our heterogeneous patient population requires further validation using a more clinically and temporally uniform ENKTL cohort.

Conclusions

Using targeted NGS and IHC profiling, we showed that activation of the JAK/STAT pathway, partly induced by gain-of-function *STAT3* mutations, is common in ENKTLs and associated with high CD30 expression. We also identified the prognostically relevant *BCOR*-MYC association. ENKTLs with high MYC expression are enriched in those with *BCOR* mutations and exhibit shorter OS, highlighting the potential utility of IHC for MYC for risk stratification. Further multiomics studies incorporating transcriptomic and epigenomic analyses should be conducted in larger ENKTL cohorts to delineate the functional consequences of MYC overexpression.

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Authorship

Contribution: N.O., K. Mochizuki, and T.K. conceived and designed the study; N.O. performed experiments, analyzed data, and wrote the paper; T.S. supervised the NGS mutational analysis; K. Miyake, N.O., A.S., M.M., I.K., K.K., A.L.F., and N.N. reviewed the cases and collected clinical information; N.O. designed the research and drafted the paper; and A.S. and A.L.F. helped in writing the paper.

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