



ORIGINAL ARTICLE

Immunophenotyping of Nodal Peripheral T-cell Lymphomas and its Association with Epstein-Barr Virus

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ABSTRACT

Background: Immunophenotyping in the rare group of nodal Peripheral T-cell Lymphomas (PTCL) exposes interesting features such as T-cell marker downregulation and paradoxically, the presence of reactive, clustered large-sized CD20 positive B-cells (B-cell proliferation). Epstein-Barr virus (EBV) has been suggested as a putative etiology in pathogenesis of B-cell lymphoma. We aimed to review the immunohistochemical profile of patients with nodal PTCL with emphasis on T-cell markers and immunophenotypic aberrations, CD20 positive large B-cells, Ki-67 scores (as a measure of proliferation index) and to assess the association of Epstein-Barr virus in various subtypes of nodal PTCL. **Methods:** 80 cases of nodal PTCL diagnosed during January 2008-June 2013 were included in the study. Relevant clinical and hematological data were collected. Using Streptavidin-Biotin-Peroxidase system, staining for CD2, CD3, CD4, CD5, CD7, CD8, CD20, EBV-LMP1 and Ki-67 were performed on all blocks. CD10, CD23, Bcl-6, CD30 and ALK-1 were used in relevant cases.

Results: 95% of patients had downregulation of at least one T-cell marker (maximum: CD7 (87%), minimum: CD3 and CD5- 9% each). 29 patients (36%) showed markers of B-cell proliferation. Only five patients (6%) were positive for EBV-LMP1. There was a significant association between EBV-LMP1 positivity and B-cell proliferation ($P=0.002$). 17 patients (21%) had high Ki-67 index ($\geq 80\%$).

Conclusion: Nodal PTCL showed frequent downregulation of T-cell markers. EBV was only infrequently positive in these Lymphomas. Clusters of large B-cells need to be noted in pathology reports and EBV needs to be tested for in such cases.

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Introduction

Nodal Peripheral T-cell Lymphomas (PTCL) comprise a heterogeneous group of mature T-cell lymphomas that include: Peripheral T-cell Lymphoma-not otherwise specified (PTCL-NOS), Anaplastic Lymphoma Kinase positive Anaplastic Large cell Lymphoma (ALK+ALCL), Anaplastic Lymphoma Kinase negative Anaplastic Large cell Lymphoma (ALK-ALCL) and Angioimmunoblastic T-cell lymphoma (AITL).¹ The ALK-ALCL is a provisional entity as per WHO (2008), while the rest are

well recognized.²

Nodal PTCL has an increased prevalence in Asian nations as well as in Southern India.³⁻⁵ They are characterized by immunophenotypic aberrations of T-cell markers.⁶⁻⁹ Some aberrations such as CD7 downregulation may offer useful diagnostic clues. The prognostic role of downregulation of individual markers and the number of downregulated markers appear to be an interesting field to explore. Another feature which is worth studying is the presence of clusters of reactive CD20 positive B-cells (B-cell proliferation)

in a background of nodal PTCL.¹⁰ It is postulated that B-cell non-Hodgkin lymphoma may originate from B-cell proliferations in patients with nodal PTCL.

Etiological agents in pathogenesis of nodal PTCL are still unknown, but for AITL, Epstein-Barr virus (EBV) is observed to play a prominent role.¹¹⁻¹³ Sporadic reports indicating evidence of positivity for EBV surrogate markers in other lymphoma subgroups; however rare, have been stated. Given the ubiquitous nature of the virus, its ability to contribute to the hematological malignancies and the fact that T-lymphocytes are responsible for response to viral antigens, a need to explore EBV as a putative viral carcinogen in nodal PTCL appears appropriate.

Our study intended to assess immunohistochemistry (IHC) of tissues diagnosed pathologically as nodal PTCL, giving special attention to aberrations, B-cell proliferation markers, Ki-67 index and to trace EBV as a potential causative agent using EBV-LMP1 antigen assay.

Materials and Methods

In this study, 91 cases of nodal PTCL were diagnosed in the Pathology Department of JIPMER, Puducherry during January 2008-June 2013. Eleven cases were

excluded subsequently as a result of review of the initial diagnosis (two cases) and technical factors (nine cases). Hence, the final sample size of the study was 80.

Relevant clinical data were collected from archives of the Departments of Pathology, Medical Oncology and the Medical Records Department. Clinical stage was determined as per Cotswold modification of Ann Arbor staging system.^{14, 15} Wherever data was not available or unsuitable for analysis, it was suitably coded for exclusion.

For cases diagnosed since December 2011, sections of 2 to 3 micron thickness were made from the formalin fixed blocks and stained with hematoxylin and eosin (H&E) as per standard operating protocol. For archival cases, the retrieved slides were screened in every case. Fresh H&E and IHC slides were prepared when necessary. Immunophenotyping for CD2, CD3, CD4, CD5, CD7, CD8, CD20, EBV-LMP1 and Ki-67 was performed in all cases. CD10, CD23 and Bcl-6 were studied in cases of AITL and CD30 and ALK-1 in patients with ALCL. Appropriate positive (known positive control tissues) and negative controls (excluding primer addition) were used (Table 1). IHC was performed manually using standard Biotin-Streptavidin-Horseradish peroxidase

Table 1: Characteristics of the antibodies used for immunophenotyping of patients with nodal PTCL

Antibody	Manufacturer	Clone	Source	Control	Positivity		Comments
					Location	Proportion	
CD2	DakoCyt®, Glostrup, Denmark.	AB75	MM [#]	Tonsil	Membrane	30%	For any case, the most strongly expressed marker is labeled as “positive” and the rest as “downregulated” with weak or no expression.
CD3	Biogenex, Fremont, USA.	PS1	MM	Tonsil	Membrane	30%	
CD5	Scytek, Logan, USA.	4C7	MM	Tonsil	Membrane	30%	
CD7	DakoCyt, Glostrup, Denmark.	CBC.37	MM	Thymus	Membrane	30%	
CD4	”	4B12	MM	Tonsil	Membrane	30%	>30% membranous expression is taken as positive.
CD8	”	C8/144B	MM	Spleen	Membrane	30%	
CD10	”	56C6	MM	Bile canaliculi	Cytoplasmic	5%	Done in suspected AITL [#] cases only.
CD20	”	L26	MM	Tonsil	Membrane	Not rigid	Noted when seen in clusters of large cells.
CD23	”	SP23	MR* [*]	Tonsil	Membrane	Not rigid	To look for distorted follicular centers.
CD30	”	Ber-H2	MM	Tonsil	Membrane / Cytoplasmic with Golgi zone accentuation	Not rigid	Done in ALCL cases only.
ALK-1	”	ALK1	MM	Known positive case of ALK+ALCL ^α	Cytoplasmic or Nuclear	Not rigid	Done in ALCL cases only.
Bcl-6	”	PG-B6P	MM	Tonsil	Nuclear	Not rigid	Done in AITL cases only.
EBV-LMP1	Guartett, Berlin, Germany.	IG6	MM	Known positive HL ^β	Cytoplasmic	Even one cell	Done in all cases
Ki-67	DakoCyt, Glostrup, Denmark.	MIB-1	MM	Known positive BL ^γ	Nuclear	Not rigid	Stratified as high (≥80%) or low

^αDakoCyt: DakoCytomation; [#]MM: Monoclonal Mouse; ^{*}MR: Monoclonal Rabbit; ^αALK+ ALCL – Anaplastic Lymphoma Kinase positive Anaplastic Large Cell Lymphoma; ^βHL: Hodgkin Lymphoma; ^γBL: Burkitt Lymphoma; [#]AITL – Angioimmunoblastic Lymphoma

method. Antigen retrieval was performed in Citrate buffer (1mmol/L, pH 6.4) solution by heating. For nuclear markers (Ki-67 and Bcl-6), microwaving for 20 minutes at 95-99°C was followed while antigen extraction by pressure cooking for 10 minutes was done for other markers. The reaction was subsequently developed with Diaminobenzidine chromogen. CD2, CD3, CD5 and CD7 are pan-T cell markers expressed in all T-cells. For interpretation, the marker with strongest positivity among these four markers is considered as “positive” and the rest three as “downregulated”. “Downregulation” can be in two forms: “weak” expression with reduced positivity or “negative” with no positivity. CD4 and CD8 can only be positive or negative, depending on the extent of expression, “downregulation” does not occur unlike the other pan-T markers. Membrane positivity of CD20 was considered positive. Cases with CD20 positive cells exceeding 30% of entire tumor were to be excluded, since it might represent a B-cell lymphoma.¹⁶ Large reactive B-cells were specifically looked for and positivity was defined, in our study, as at least one cluster of five or more CD20 positive cells intermixed with the Lymphoma cells.¹⁰ CD10 was taken as positive if at least 5% of the neoplastic cells expressed the marker.¹⁷ Ki-67 index was quantified by manually counting the number of positive cells among all cells in an area with highest intensity. A cut-off $\geq 80\%$ was considered as high based on a prior study.¹¹ A brief summary on interpretation of all IHC markers used in the study is provided in Table 1.

Statistical analysis was performed using SPSS software

(Version 16, SPSSInc, Chicago, IL). Chi-square (χ^2) test or Fisher exact test were used for studying the association between categorical variables. A P value less than 0.05 was considered statistically significant. All steps and procedures performed in the current study were approved by Institute Review Board (Letter number: SEC/2011/4/31) in accordance with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants of the study.

Results

During the study period of January 2008-June 2013, 89 cases of nodal PTCL were diagnosed which comprised 18% of all cases diagnosed with lymphoma, 25% of NHLs and 75% of T-cell NHLs. Eventually, only 80 samples were analyzed for IHC.

There were only three cases where all pan T-cell markers (CD2, CD3, CD5, CD7) were equally and strongly positive. The rest of the samples had a downregulation of at least one of the above-mentioned markers (Table 2). While the majority of the ALCL cases showed loss of two of the four T-cell markers tested (48% in ALK+ ALCL and 74% in ALK- ALCL), the majority of cases in PTCL-NOS and AITL showed loss of one T-cell marker only. Loss of all four T-cell markers were seen in two patients whose pathology was reported as null cell ALCL. Thus, marker downregulation was more common in ALCL subtypes.

A summary of T-cell marker expression is shown in Table 3. CD2 was downregulated in 52% of patients with

Table 2: Percentage of patients in relation to the number of downregulated pan-T cell markers* among the subtypes of Nodal Peripheral T-cell Lymphoma

Number of pan-T markers downregulated	% of patients (n=number of patients)				Total (n=80)
	PTCL- NOS (n=41)	ALK+ALCL (n=23)	ALK-ALCL (n=8)	AITL (n=8)	
0	5	0	13	0	4
1	61	34	13	63	49
2	24	48	74	37	38
3	10	9	0	0	7
4	0	9	0	0	2

* The four pan-T cell markers include CD2, CD3, CD5 and CD7; PTCL-NOS: Peripheral T-cell Lymphoma, Not Otherwise specified, ALK+/- ALCL – Anaplastic Lymphoma Kinase positive / negative Anaplastic Large Cell Lymphoma, AITL – Angioimmunoblastic Lymphoma

Table 3: Percentage of patients with positive and downregulated T-cell markers across the four subtypes of Nodal Peripheral T-cell Lymphoma

DIAGNOSIS (n=number of patients)	Marker expression (expressed in % of patients)															
	CD2		CD3		CD4		CD5		CD7		CD8					
	P	D	P	D	P	N	P	D	P	D	P	N				
	W	N	W	N	W	N	W	N	W	N	W	N				
PTCL-NOS(n=41)	56	7	37	93	2	5	41	59	96	2	2	15	68	17	76	24
ALK+ALCL(n=23)	30	0	70	78	0	22	22	78	83	0	17	9	39	52	61	39
ALK-ALCL(n=8)	38	0	62	75	0	25	0	100	100	0	0	12	88	0	50	50
AITL (n=8)	63	0	37	100	0	0	13	87	88	0	12	13	62	25	88	12
Total (n=80)	48	3	49	91	1	8	29	71	91	1	8	13	61	26	70	30

P: Positive; D: Downregulated; W: Weak; N: Negative; PTCL-NOS – Peripheral T-cell Lymphoma, Not Otherwise specified, ALK+/- ALCL – Anaplastic Lymphoma Kinase positive / negative Anaplastic Large Cell Lymphoma, AITL – Angioimmunoblastic Lymphoma

Table 4: Comparison of patterns involving various combinations of CD4 and CD8 staining among the four subtypes of Nodal Peripheral T-cell Lymphoma

DIAGNOSIS (n=number of patients)	% of patients			
	CD4+/CD8+	CD4+/CD8-	CD4-/CD8+	CD4-/CD8-
PTCL-NOS (n=41)	49	2	44	5
ALK+ALCL (n=23)	26	9	39	26
ALK- ALCL (n=8)	0	0	75	25
AITL (n=8)	25	0	63	12
Total (n=80)	35	4	47	14

PTCL-NOS – Peripheral T-cell Lymphoma, Not Otherwise specified, ALK+ / - ALCL – Anaplastic Lymphoma Kinase positive / negative Anaplastic Large Cell Lymphoma, AITL – Angioimmunoblastic Lymphoma

Table 5: Ki-67 cell proliferation index expression across the four subgroups of Nodal Peripheral T-cell Lymphoma

DIAGNOSIS (n=number of patients)	Ki-67 index (%)		% of patients with high Ki-67 index*
	Median	Range	
PTCL-NOS (n=41)	20	5-95	17
ALK+ALCL (n=23)	8	5-90	26
ALK-ALCL (n=8)	32.5	5-90	25
AITL (n=8)	20	5-85	25
Total	20	5-95	21

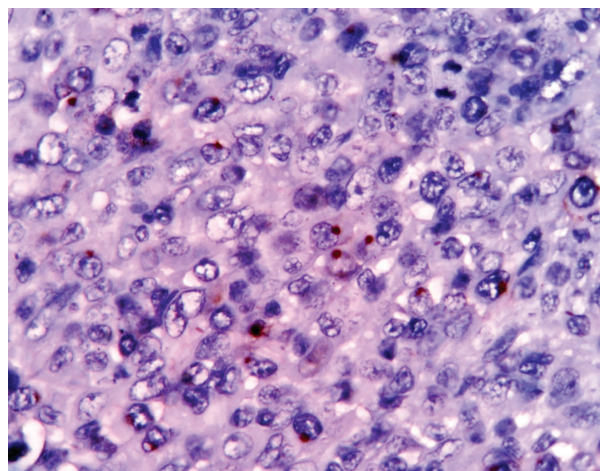
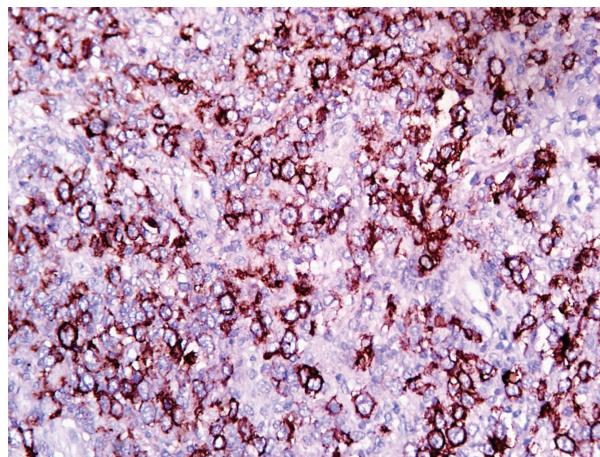
*High Ki-67 index is defined as a value of $\geq 80\%$ as defined by Went *et al.* [11]; PTCL-NOS – Peripheral T-cell Lymphoma, Not Otherwise specified, ALK+ / - ALCL – Anaplastic Lymphoma Kinase positive / negative Anaplastic Large Cell Lymphoma, AITL – Angioimmunoblastic Lymphoma

nodular PTCL, maximally in ALCL-ALK positive cases (70%). CD3 was downregulated in only 9% (seven cases) of cases of nodal PTCL. In these seven cases, CD5 was found to be strongly positive in five and the remaining two cases were representing the null cell variant of ALCL. CD5 was the most consistently positive marker being positive in at least 83% of all subgroups. It was downregulated in only six patients, two of whom were null cell ALCL-ALK positive patients. CD7 was the most frequently downregulated marker overall. CD4 was negative in 71% while CD8 was positive in 70% of all nodal PTCL. Taken together in our study, CD-/CD8+ was the dominant combination seen in 47% of all nodal PTCL samples and in all subgroups except in PTCL-NOS, where CD4+/CD8+ was more common (49%) (Table 4).

To ascertain if the downregulation of T-cell markers could predict an advanced stage (stage IV disease), an attempt was made at statistical association. When individual T-cell markers were tested for correlation with stage III and IV, it was seen that only CD8 downregulation showed statistically significant correlation with Stage IV disease in nodal PTCLs ($P=0.04$). The number of lost markers did not correlate either with advanced stage of the disease (Stages III and IV taken together) in nodal PTCLs or with any of its four individual subtypes.

All the ALCL patients had CD30 positivity and the division into ALK positive and ALK negative groups was done on the basis of IHC staining for ALK protein. A 'dot' or Golgi zone positivity of neoplastic cells helps corroborate the diagnosis (Figure 1).

In AITLs, among the neoplastic cells, CD10 positivity was seen in 63% cells compared to 75% by Bcl-6. Proliferation of follicular dendritic cell network as seen by expanded and distorted follicular centers, highlighted by CD23, was seen in 88% of the patients.

**Figure 1:** Dot positivity for CD30 in Golgi region of the neoplastic cells in a case of ALK positive Anaplastic Large T-Cell Lymphoma. (IHC, 400 \times)**Figure 2:** Clusters of large CD20 positive cells in a focus of Anaplastic Large T-cell Lymphoma. (IHC, 100 \times)

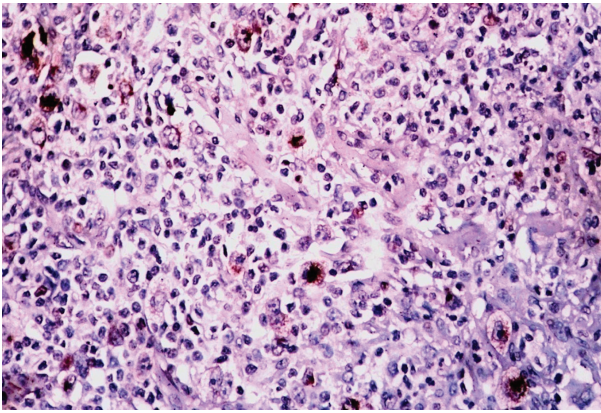


Figure 3: Cytoplasmic positivity for EBV-LMP1 in a case of Angioimmunoblastic T-cell Lymphoma. (IHC, 100×)

B-cell proliferation was seen in 36% (29 patients) with a maximum of 63% in AITL (Figure 2). 64% of patients with B-cell proliferation markers had stage IV disease compared to 44% in those who did not. The correlation was *not* statistically significant ($P=0.1$). Follow up data was unfortunately available for only 11 of these patients of which two patients (18%) had died of the disease. In contrast, 53% of patients without B-cell proliferation had died or were keeping unwell at last follow-up.

EBV-LMP1 positivity was observed in five cases; two cases each were of PTCL-NOS and AITL subtypes, while one case was an ALK positive ALCL (Figure 3). There was one patient each in Stage I and II, two patients in Stage IV and one could not be staged due to incomplete workup. Three patients are currently doing well, while for two patients the current status is unknown due to lack of follow up. All five patients showing EBV positivity demonstrated proliferation of reactive CD20 positive cells. The correlation between EBV-LMP1 positivity and the presence of CD20 positive B-cell clusters was significant ($P=0.002$).

Ki-67 index showed a wide range in nodal PTCL (Table 5). The average tumor proliferation index, calculated as “median” was 20%. It was maximum in ALK negative ALCL with a median of 32.5%. Overall 21% of the patients had a “high” Ki-67 index defined as a value of $\geq 80\%$.¹¹ Higher index for Ki-67, as expected, were observed in cases of nodal PTCL with Stage IV disease ($P=0.041$).

Discussion

As already pointed out, nodal PTCL are characterized by immunophenotypic aberrations.⁶⁻⁹ In fact, 96% of nodal PTCL patients had loss of at least one of the four pan T-cell markers which are CD2, CD3, CD5 and CD7. Among our patients, 95% of those who were categorized as PTCL-NOS had an aberrant phenotype. In addition, all patients with AITL had downregulation of T-cell markers. This figures have been reported as 80% and 96% downregulation of T-cell markers in PTCL-NOS and AITL patients, respectively in previous studies.^{8, 18} Loss of CD2 as a pan T-cell marker was the second most common marker downregulated among all subgroups.^{1, 6}

CD3 and CD5 were both positive in 91% of nodal PTCL cases. CD3 is a marker with high sensitivity in nodal

PTCL except for ALCL, where they can be negative in more than 75% of the cases.^{19, 20} We found about one-fourth of the ALCL patients showing downregulation of CD3. Similarly, CD5 is said to be rarely lost in PTCL-NOS and AITL except one contradicting study by Went et al.^{1, 11} The same was reflected in our study where CD3 was negative in only nine cases. In all these cases CD5 was positive. Similarly in samples of eight patients negative for CD5, CD3 helped in making the diagnosis. We can therefore conclude that CD3 and CD5 complement each other as a diagnostic panel.

CD7 is the most frequently lost marker in T-cell Lymphomas; especially, in PTCL-NOS.^{6, 8} Our study mirrored the same observation with downregulated CD7 in 87% of all nodal PTCL patients. The importance of this finding is however debatable. As mentioned earlier, CD7 can be decreased in reactive conditions, particularly in the skin. Also, CD7 shows weak expression in paraffin embedded blocks. Loss of CD3 or CD5 is more significant and supportive of a T-cell neoplasm or a NK-cell proliferation/neoplasm than CD7 downregulation.²¹ Most of the nodal T-cell lymphomas exhibit a CD4+/CD8-immunophenotype.^{1, 22-26} In contrast, our study showed that CD4-/CD8+ pattern was the commonest among all subgroups except for the PTCL-NOS (double positive pattern was the commonest) (Table 4). Only 4% (three cases) of our samples demonstrated the CD4+/CD8- pattern. The explanation for these findings could be technical factors and so these results need to be confirmed in future studies. Procedural lapses are unlikely since assessment for other markers performed on the same setting had taken up the stain correctly. Poor quality stain (control CD4 slide showed mostly weak positivity) and a heat-induced antigen damage (many sections were stored for up to three months at room temperature before the staining) may explain the unexpected results. Otherwise, the observed pattern may simply imply a geographic variant that needs to be verified by further studies and reports.

The attempted correlation of pan T-cell markers with advanced stage of the disease did not yield significant results perhaps due to the limited number of the cases in the study. However, CD8 downregulation correlated with Stage IV disease in patients with nodal PTCL. Since, significance was lost in individual subgroups, the practical utility of this finding is questionable. To the best of our knowledge, association between advanced stage disease and marker downregulation has not been assessed before, but may serve as a useful predictor of prognosis in lymphoma.

CD10 is reported to be positive in 30-90% of all cases of AITL.^{10, 18, 27, 28} In this study, staining for CD10 in AITL was positive in 63%. Bcl-6 positivity in our study was 76%, similar to that observed by Dupuis et al (75%).²⁹

EBV-LMP1 positivity was seen in only 6% of our patients by IHC. This makes EBV unlikely as a causative role in the pathogenesis of nodal PTCL. Of note, EBV detection by LMP1 method is not considered sensitive. This method is reported to be able to detect the virus in about 41-83% of the cases that were positive by EBV-encoded RNA In Situ Hybridization (EBER-ISH).^{12,}

^{13, 30} Thus, the real frequency of positivity for EBV in lymphoma may be actually higher. d'Amore et al found EBV positivity to be an unfavorable prognostic factor.³¹ As observed by Tan et al, we also found a significant association between EBV-LMP1 positivity and CD20 positive large B-cell clusters (P=0.002).¹⁰ These large cells are considered as proliferating immunoblasts that are prone to somatic hypermutation and development of B-cell lymphoma in the presence of viral triggers. Since all the EBV-LMP1 positive patients in our study had clusters of large CD20 cells, it would be prudent to follow EBV positive patients for long term for development of B-cell Lymphoma.

CD20 positivity in large B-cells was seen in 36% of the patients. Tan et al. found a frequency of 19% for PTCL-NOS as well as AITL patients to harbor B-cell proliferation, while Lome-Maldonado et al found an incidence of 18% in AITL patients.^{10, 32} Of note, statistical significance was not observed between CD20 positivity and staging of the disease. To reach any possible meaningful interpretation of statistical significance of this proliferation on clinicopathological profile and prognosis, larger data would be required.

Ki-67 has been traditionally used to indicate a poor prognosis in malignancies. Went et al proposed a high Ki-67 index of $\geq 80\%$ in a clinicopathological scoring system. In their study, 11% of PTCL-NOS and 5% of AITL had such high Ki-67 scores.¹¹ In our study; using the same cut off, more patients (21%) presented with higher index and correlated with Stage IV disease (P=0.041).

The low positivity rate for the virus to be detected in tissues is likely to be due to the unavailability of EBER-ISH, a very sensitive technique. Various technical factors appear to have given rise to apparently discordant IHC results. Overall, small number of patients in some subgroups due to poor follow up prevented a precise statistical analysis of survival.

Conclusion

Immunophenotypic aberrations are a reality in patients with nodal PTCL and attempts should be made to understand and utilize this phenomena for assigning prognostic significance.

Since EBV-LMP1 is not sensitive, EBER-ISH is suggested to assess EBV positivity. In addition, we suggest considering the patients with either EBV-LMP1 positivity and/or evidence of B-cell proliferation under close follow-up.

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Conflict of Interest: None declared.

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