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Detection of the 30-bp deletion and protein expression of Epstein-Barr virus latent membrane protein 1 in extranodal NK/T cell lymphoma and its clinicopathological significance

Xingmei Lu[1*], Qingsong Han[2], Peng Li[1], Kate Huang[1], Xiuhuan Ji[1], Suidan Chen[1], Rixu Lin[1] and Xiaoyu Wang[1]

Abstract

Background Extranodal natural killer/T-cell lymphoma (ENKTCL) is strongly associated with Epstein-Barr virus (EBV) infection. A 30-base-pair deletion in latent membrane protein 1 (del-LMP1) represents the most common variant in the EBV genome, but its clinicopathological significance in ENKTCL remains poorly elucidated. Some scholars suggested that the LMP1 protein product carrying the deletion gene reduced immunogenicity, allowed it to escape immune surveillance in immunocompetent hosts and confer a survival advantage. Therefore, simultaneous assessment of del-LMP1 and LMP1 protein expression may provide deeper insights into the potential role of LMP1 in ENKTCL tumorigenesis and progression. This study aimed to investigate the impact of del-LMP1 and LMP1 protein expression on the clinicopathological manifestations and prognosis of ENKTCL patients in Wenzhou.

Methods The clinical and histological characteristics of 42 ENKTCL cases were retrospectively evaluated. Del-LMP1 was detected using a nested polymerase chain reaction and Sanger sequencing, while LMP1 protein expression was assessed via immunohistochemistry. Overall survival (OS) was analyzed.

Results The LMP1 gene was identified in 37/42 ENKTCL cases, including 2 wild-type (wt-LMP1), 35 del-LMP1 cases. LMP1 protein expression was positive in 21/42 cases. In the control group, the LMP1 gene was detected in 6/10 cases, all of which were del-LMP1, and the LMP1 protein was positive in 4/10 cases. Fisher's exact test revealed no significant differences between the two groups in the LMP1 gene, del-LMP1, or LMP1 protein expression. Additionally, there was no significant correlation between del-LMP1 and LMP1 protein expression and clinical characteristics such as age, gender, or vascular invasion. However, LMP1 protein expression was significantly higher in necrotic tissues ($p=0.030$) and younger patients with del-LMP1 ($p=0.004$). Survival analysis showed no significant difference in OS between wt-LMP1 and del-LMP1 patients ($p=0.331$) or between LMP1-positive and -negative cases ($p=0.592$).

Conclusion In this retrospective cohort, we demonstrated that del-LMP1 might be the predominant variant rather than a phenotype-associated polymorphism in ENKTCL from a molecular epidemiological perspective. Moreover, LMP1 protein expression was associated with necrotic tissue and younger patients with del-LMP1, possibly due to the enhanced pathogenic effect of the mutated LMP1 isolate protein.

*Correspondence:
Xingmei Lu
luxingmei@wmu.edu.cn
Full list of author information is available at the end of the article

Keywords Extranodal natural killer/T-cell lymphoma (ENKTCL), Latent membrane protein 1 (LMP1), Epstein-Barr virus (EBV)

Background

Extranodal natural killer/T-cell lymphoma (ENKTCL) is a rare subtype of EBV-associated non-Hodgkin lymphoma with a highly aggressive clinical course [1]. It has a predilection for extranodal involvement, especially in the upper aerodigestive tract (UADT), but also affects the skin, gastrointestinal tract, soft tissue, and testis [2]. Histologically, ENKTCL is characterized by angioinvasion and angiodestruction, accompanied by prominent coagulative necrosis and a cytotoxic phenotype [1]. ENKTCL predominantly occurs in East Asia and Latin America rather than in Europe and North America [3]. Although its pathogenesis is still unclear, the effect of the strong geographic distribution of ENKTCL on specific populations suggests a genetic predisposition for ENKTCL.

Epstein-Barr virus (EBV), a member of the Herpesviridae family, infects approximately 95% of the world's population and persists as an asymptomatic, life-long infection [4]. The importance of EBV in ENKTCL lymphomagenesis was first recognized in 1990 and has been confirmed in various studies since then [2, 5–8]. Further studies have focused on the viral proteins and their genetic variants to investigate the role of EBV in this lymphoma's pathogenesis [2, 4, 9].

Latent membrane protein 1 (LMP1) is one of the important oncogenes of EBV carcinogenicity and has the ability to induce malignant transformation in epithelial and B cells [10, 11]. The LMP1 gene has been shown to have polymorphisms, among which the 30-base-pair (bp) deletion (del-LMP1) is the most common variant in the C-terminus and was first detected in nasopharyngeal carcinoma (NPC) patients from southern China [12, 13]. It occurs at the 3' end of the C-terminal tail and is associated with the CTAR2 functional domain. However, there were conflicting views about the significant role of the 30-bp deletion in conferring the more tumorigenic potential of the LMP1 gene [6, 14–21]. Knecht et al. [15] reported an association of del-LMP1 with cases of European Hodgkin's disease (HD) and suggested that del-LMP1 was associated with aggressive histology or behavior; however, a study from Mexico held a negative attitude and disputed a definite pathogenetic role for del-LMP1 in ENKTCL [6]. Most reports of del-LMP1 have suggested that this variant is widespread worldwide but varies in different geographic regions [10, 22–24]. A recent review by Montes-Mojarro et al. [2] analyzed the distribution of LMP1 variants in 6 studies involving 140 ENKTCL patients showed a clear predominance of the wild-type LMP1 (wt-LMP1) (52.1%) with 37.1% of patients harboring del-LMP1 [6, 25–28]. While, other authors have found a high frequency of del-LMP1 detected in Asian ENKTCL patients [29–33]. However, to the best of our knowledge, the clinicopathological significance of del-LMP1 in ENKTCL has not been fully elucidated to date.

Previous studies have found that B-cell-derived LMP1 isolate protein is highly immunogenic [34]. Kingma et al. [35] suggested that mutations in the LMP1 gene reduced the immunogenicity of LMP1 and thus escaped immune surveillance in immunocompetent hosts. Therefore, simultaneous assessment of LMP1 expression at the protein level may contribute to our better understanding of the underlying significance of LMP1 in ENKTCL tumorigenesis and development. Herein, we conducted a retrospective cohort study to investigate whether 30-bp deletion of the LMP1 gene and LMP1 protein expression play any role in the clinicopathological manifestations and prognosis of ENKTCL in Wenzhou.

Materials and methods

Sampling and data collection

We retrospectively reviewed records and samples of ENKTCL from 125 patients between January 2016 and June 2022 at the Department of Pathology, the First Affiliated Hospital of Wenzhou Medical University. The ENKTCL diagnosis was according to the World Health Organization's (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues [1]. Patients with other malignancies, patients exposed to neoadjuvant chemotherapy and radiation therapy before surgery, and patients with incomplete data were excluded. We selected 42 cases with adequate tissue material for further investigation using nested polymerase chain reaction (PCR) and immunohistochemistry (IHC). Patients' clinical information was collected from electronic medical records. In addition, 10 nasopharyngeal chronic inflammation tissues were selected as the control group. The presence of EBV was confirmed by in situ hybridization (ISH) for EBV-encoded small RNA (EBER) (Zhongshan Golden Bridge Company, Beijing, China).

DNA extraction

Fifty-two eligible samples (42 ENKTCL and 10 control tissues) were obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks by cutting 5-μm-thick sections. Each specimen used a fresh microtome blade to

reduce the risk of cross-contamination. According to the manufacturer's protocol, deoxyribonucleic acid (DNA) was extracted and purified from samples using the Biospin FFPE Tissue Genomic DNA Extraction Kit.

Nested PCR for detection of the LMP1 gene

Nested PCR was performed to amplify the C-terminus of the LMP1 gene. The first round of amplification was done using the outer primers of LMP1-F1 (5'-ATTGGCACA AGATGGAAAGC-3') and LMP1-R1 (5'-TCCTTTGGC TCCTCCTGTTT-3') in a total volume of 20 μl containing Taq PCR Master Mix 12.5 μl, 1 μl of DNA extracted from tissues, 1 μl of each primer, and 4.5 μl deionized sterile water (ddH2O). The cycling protocol was started with an initial denaturation for 5 min at 98℃, then 30 cycles of denaturation for 30 s at 95 ℃, annealing for 30 s at 54 ℃, and extension for 75 s at 72 ℃. A final elongation step for 10 min at 72 ℃ was then conducted. For the second round of PCR, we employed the inner primers of LMP1-F2 (5'-GAGGGAGAGTCAGTCAGGC-3') and LMP1-R2 (5'-AGACGGAAGAGGTTGAAAAC-3'); then 1 μl of the PCR product was used as the template. The second round utilized similar experimental conditions except for the extension time, which was 30 s. In each experiment, positive and negative controls were conducted, respectively. The primers were designed according to the standard strain EBV 95.8 and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Agarose gel electrophoresis

The nested PCR products were separated by electrophoresis on a 1.2% agarose gel in 1× Tris-Acetate-EDTA (TAE) buffer for 45 min. The gel was stained with 4S green nucleic acid gel stain and photographed under ultraviolet light. The DNA molecular weight standard (100-5000) was used as the marker.

DNA sequencing

The specific band of interest was excised from the gel and purified using the Agarose Gel DNA Extraction Kit (Sangon Biotech Co., Ltd., Shanghai, China) following the manufacturer's protocols. The PCR products were sequenced by Sangon Biotech Co., Ltd., a service provider, Sanger sequencing method was used to determine the presence of deletion mutations, and the sequencing primer was LMP1-R2. The results were analyzed using MegAlign software.

IHC for the detection of LMP1 protein

IHC was performed on sections of 3.5 μm thickness. After being deparaffinized in xylene and rehydrated using a graded alcohol series, the antigen retrieval was followed in an EDTA buffer at 95 ℃ for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature. Slides were then incubated with mouse anti-LMP1 monoclonal antibody (clone CS1-4, MXB, Fujian, China) overnight at 4 ℃ and horseradish peroxidase (HRP)-labeled secondary antibody for 20 min at room temperature. Diaminobenzidine was used as the chromogen for the immunostaining. Finally, the sections were counterstained with hematoxylin. EBV-positive Hodgkin's lymphoma sections were performed as positive controls according to the manufacturer's instructions. Negative controls were obtained by substituting the primary antibody with phosphate-buffered saline. LMP1-positive was determined when the lymphoma cells were positive according to the methods described by Kume et al. [17].

Statistical analysis

Statistical analyses were performed with SPSS 26.0 software. The Chi-squared test and Fisher's exact test were used to compare variables among groups (calculated categorical variables). Overall survival (OS) was calculated from the date of initial diagnosis until death from any cause or the date of last follow-up. Survival curves were calculated with the Kaplan-Meier method, and statistical significance was assessed using the log-rank test. All statistical tests were two-sided; a p-value ≤ 0.05 was considered statistically significant.

Results

Patients characteristics

The characteristics of 42 ENKTCL patients were summarized in Table 1. There were 31 men and 11 women with a male-to-female ratio of 2.8 to 1. The age range was from 21 to 87 years, with a median age of 55 years and 16 patients over 60 years old. The most common site of occurrence was the UADT ($n=28$); epistaxis, rhinorrhea, and nasal obstruction were initial manifestations. Thirteen patients had B symptoms, and eighteen had elevated serum lactate dehydrogenase (LDH) levels. Thirty-two patients were classified as stages I/II according to the Ann Arbor stage. Morphologically, most cases showed pleomorphic infiltrates, angiodestruction occurred in 22 samples, and variable degrees of necrosis were found in 23 tissues (Fig. 1A). CD56 (clone UMAB83, Zhongshan Golden Bridge Company, Beijing, China) and Granzyme B (clone EP230, Zhongshan Golden Bridge Company, Beijing, China) were positive in all cases. The median value of Ki-67 (clone UMAB107, Zhongshan Golden Bridge Company, Beijing, China) was 80% (range: 35%-98%). All cases showed EBER-positive signals in tumor cell nuclei (Fig. 1B).

Twenty patients had chemotherapy alone, while twelve received combined chemoradiotherapy. Three patients

Table 1 The clinicopathological characteristics of the 42 ENKTCL patients

Characteristics	No of patients (%)
Sex	
Male	31 (73.8)
Female	11 (26.2)
Age (years)	
≤60	26 (61.9)
>60	16 (38.1)
Primary site	
UADT	28 (66.7)
Extra-UADT	14 (33.3)
Ann Arbor stage	
I/II	32 (76.2)
III/IV	10 (23.8)
Vascular invasion	
Present	22 (52.4)
Absent	20 (47.6)
Necrosis	
Present	23 (54.8)
Absent	19 (45.2)
Ki-67 (%)[a]	
<80	17 (41.5)
≥80	24 (58.5)
B symptoms	
Present	13 (31.0)
Absent	29 (69.0)
LDH[b]	
Normal	18 (46.2)
Elevated	21 (53.8)

[a] Data of Ki-67 were available in 41 patients

[b] Data of LDH were available in 39 patients

received autologous stem cell transplants and three more received allogeneic stem cell transplantations. By the end of the follow-up, ten patients had died of disease. The median survival was 19.1 months (range: 0.1-48 months). The 1-year and 3-year estimated survival rates were 79.2% and 69.3%, respectively.

Detection of the LMP1 gene and del-LMP1

The LMP1 gene was successfully amplified from 37/42 ENKTCL cases and 6/10 non-malignant controls, as shown in Table 2. Two types of PCR products, 251 bp and 221 bp, were obtained following amplification of the LMP1 gene. Representative gel electrophoresis results were shown in Fig. 2. Sanger sequencing confirmed that the 221 bp product contained a special 30-bp deletion (from nucleotide 168255 to 168285). The results indicated that wt-LMP1 was present in 2 ENKTCL patients, while del-LMP1 was present in 35 ENKTCL patients and 6 non-malignant controls, as depicted in the relevant sequence diagram in Fig. 3. No statistical difference was found for del-LMP1 between ENKTCL and non-malignant nasopharyngeal tissues ($p=0.190$, Table 2). We analyzed correlations between del-LMP1 and clinicopathologic characteristics, including gender, age, Ann Arbor stage, B symptoms, LDH, vascular invasion, and necrosis, and all results lacked statistical significance (Table 3).

Detection of LMP1 protein expression

IHC staining was used for detecting LMP1 protein expression (Fig. 4). As shown in Table 2, LMP1 protein expression was detected in 21 out of 42 ENKTCL specimens and 4 out of 10 control samples, and the difference between the two groups was not statistically significant ($p=0.729$). We evaluated correlations between LMP1 protein expression and the clinical features of ENKTCL patients. Although most parameters were not statistically significant, LMP1 protein expression in coagulation

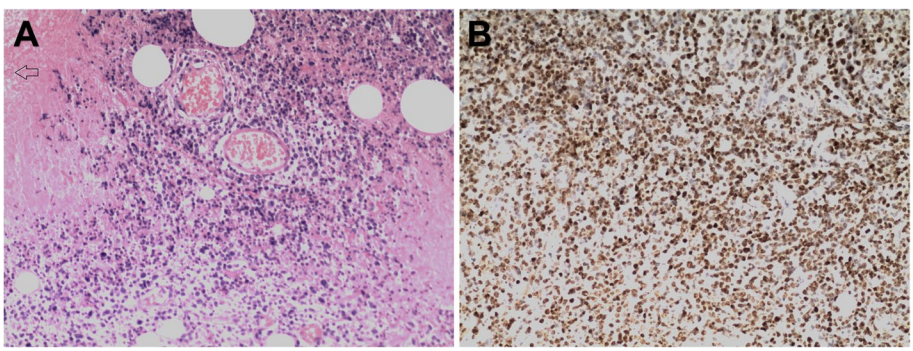

Fig. 1 Histological features of ENKTCL. **A** Morphologically, tumor cells showed pleomorphic infiltrates, angiocentricity, angiodestruction, and necrosis (empty arrow). (×100) **B** In situ hybridization of Epstein-Barr virus-encoded small RNA (EBER) showed strong positivity in most tumor cells. (×100)

Table 2 Latent membrane protein 1 (LMP1) gene, 30-bp deletion of LMP1 (del-LMP1), and LMP1 protein in extranodal natural killer/T-cell lymphoma (ENKTCL) and control tissues

	ENKTCL % ($n=42$)	Control tissues % ($n=10$)	*p*-value
LMP1 gene			
Positive	37	6	0.057
Negative	5	4	
Del-LMP1			
Yes	35	6	0.190
No	7	4	
LMP1 protein			
Positive	21	4	0.729
Negative	21	6	

necrosis tissues increased significantly ($p=0.030$), and there was a trend toward increased expression of LMP1 protein in patients with elevated LDH ($p=0.075$) and younger patients (≤ 60 years old, $p=0.057$) (Table 3).

In the del-LMP1 group, 19 cases were LMP1 protein-positive. There was no correlation between protein expression and 30-bp deletion ($p=0.489$). Table 4 demonstrated that young patients (≤ 60 years old) had significantly higher LMP1 protein expression in the del-LMP1 group than older patients (>60 years old) ($p=0.004$).

Association between del-LMP1, LMP1 protein expression, and survival outcomes

Among 35 ENKTCL cases with del-LMP1, the median survival was 18.2 months (range: 0.1-48 months), and

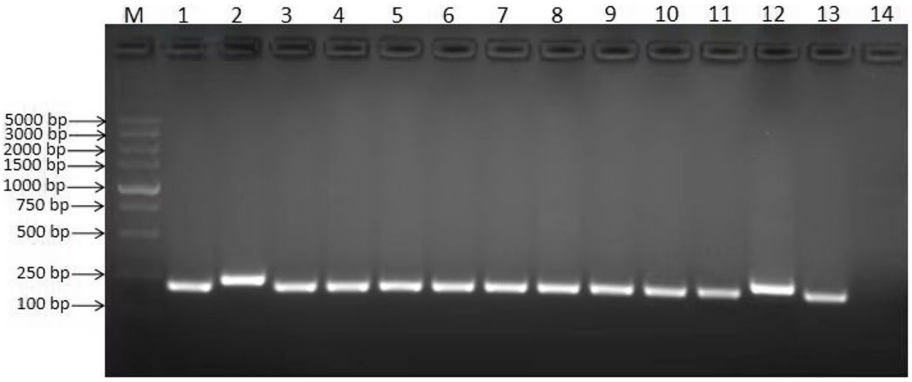

Fig. 2 DNA analysis of specimens after polymerase chain reaction amplifications of Epstein-Barr virus genome latent membrane protein 1 (LMP1). M, marker. Lanes 1 and 3-11 showed del-LMP1 products. Lanes 2 and 12 showed a 30-bp sequence preserved in the LMP1 gene. Lane 13, positive control. Lane 14, negative control

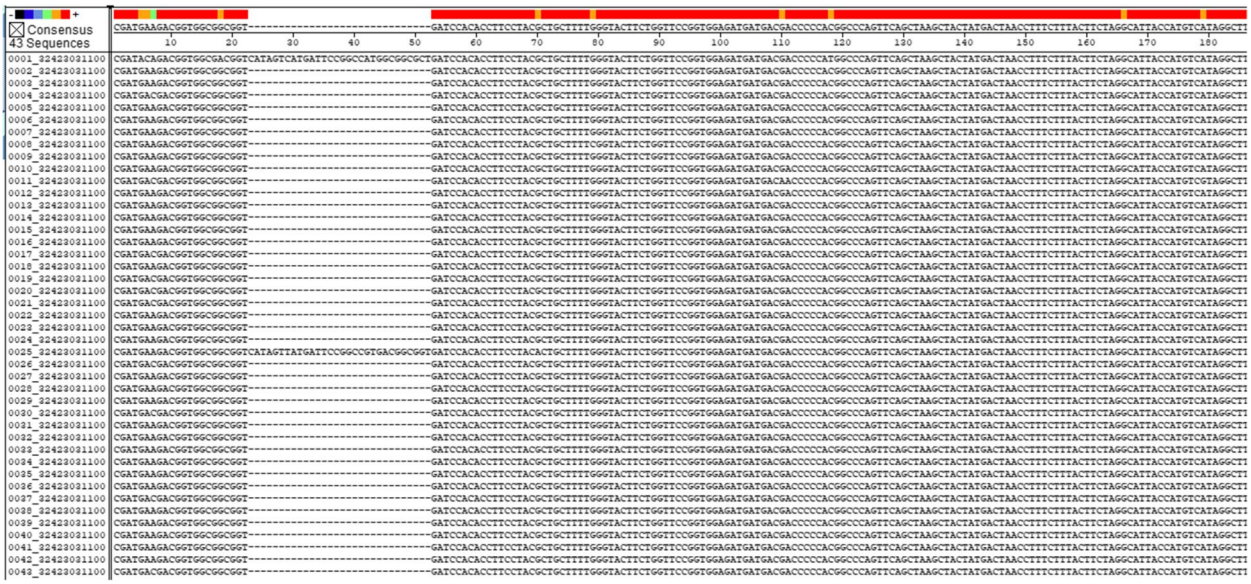

Fig. 3 Sequencing results revealed a 30-bp deletion at the same position in 35 ENKTCL patients and 6 controls, while 2 ENKTCL patients bearing the wild-type LMP1

Table 3 The relationship between latent membrane protein 1 30-bp deletion (del-LMP1) and LMP1 protein expression and clinical characteristics in patients with extranodal natural killer/T-cell lymphoma (ENKTCL)

Characteristics	Del-LMP1	*p*-value	LMP1 protein	*p*-value
Sex				
Male	28/31	0.063	16/31	0.726
Female	7/11		5/11	
Age				
≤60	22/26	1.000	16/26	0.057
>60	13/16		5/16	
Tumor site				
UADT	25/28	0.197	15/28	0.513
Extra-UADT	10/14		6/14	
Ann Arbor stage				
I/II	27/32	0.746	16/32	0.870
III/IV	8/10		5/10	
Vascular invasion				
Present	19/22	0.691	12/22	0.537
Absent	16/20		9/20	
Necrosis				
Present	21/23	0.214	15/23	0.030*
Absent	14/19		6/19	
Ki-67 (%)[a]				
<80	15/17	0.074	11/17	0.146
≥80	19/24		10/24	
B symptoms		0.176		0.426
Present	9/13		8/13	
Absent	26/29		13/29	
LDH[b]				
Normal	13/18	0.432	6/18	0.075
Elevated	18/21		13/21	

[a] Data of Ki-67 were available in 41 patients

[b] Data of LDH were available in 39 patients

*$P \leq 0.05$ was considered statistically significant

the estimated 1- and 3-year OS rates were 77.5% and 62.0%, respectively (Fig. 5A). Two cases with wt-LMP1 survived, with a follow-up period of 20 months and 42 months, respectively. There was no significant difference in the overall survival between the two groups, but it could be seen that the wt-LMP1 cases had a trend toward a better prognosis ($p = 0.331$, Fig. 5A).

The estimated 1-year survival rate was lower in the LMP1 protein-positive group (73.5%) than in the negative group (84.4%), but there was no statistically significant difference in overall survival between LMP1 protein-positive and negative cases ($p = 0.592$, Fig. 5B).

In the del-LMP1 group, there was no statistically significant difference in overall survival between LMP1protein-positive and negative cases ($p = 0.580$, Fig. 5C).

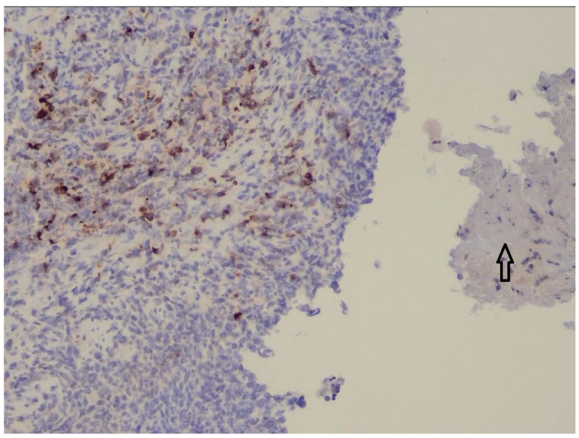

Fig. 4 Immunohistochemical analysis showed that the tumor cells were positive for LMP1, and the positive cells were stained brown, and the empty arrow marked necrotic tissue. (×200)

Discussion

ENKTCL is an increasingly recognized EBV-associated disease entity with aggressive clinical behavior and unique clinicopathological features. Due to the important role of LMP1 in immunogenicity and tumorigenicity, its genetic diversity has been extensively studied in the literature [36, 37]. Some studies have shown that LMP1 has a geographical bias [21], while others have shown that a specific variant of EBV is prevalent, causing disease and malignancy [4].

In this study, LMP1 genes were detected in 37/42 (88.10%) ENKTCL cases, compared to 6/10 (60%) in the control group. This result not only reflected the high rate of EBV infection in ENKTCL but also suggested that LMP1 may be involved in the pathogenesis and development of ENKTCL, which provided a theoretical basis for further study into its mechanisms. However, it is important note that a certain proportion of healthy carriers also harbor the LMP1 gene, indicating that EBV infection may occur before the formation of tumors. We speculated that mature resting B cells infected with EBV remain quiescent in healthy carriers and may be activated under certain conditions, leading to EBV-associated tumorigenesis [38]. In our study, the LMP1 gene was still undetectable in 5 ENKTCL cases and four control tissues. The possible reasons are: (l) The formation and progression of ENKTCL are associated with multiple factors, and the EBV LMP1 gene may not be an essential cause; (2) the LMP1 fragment may be lost during the differentiation and proliferation of lymphoma cells; (3) some cases seem to exhibit latency I [39, 40]; and (4) EBV-infected mature resting B cells do not need to express latent viral proteins to avoid immune surveillance [38].

Table 4 Association of LMP1 protein expression with clinical characteristics in extranodal natural killer/T-cell lymphoma (ENKTCL) patients with 30-bp deletion of latent membrane protein 1 (del-LMP1)

Characteristics	LMP1 protein		*p*-value
	Positive	Negative	
Sex			0.424
Male	16	11	
Female	3	5	
Age			0.004*
≤60	16	6	
>60	3	10	
Tumor site			0.713
UADT	13	10	
Extra-UADT	6	6	
Ann Arbor Stage			1.000
I/II	4	3	
III/IV	15	13	
Vascular invasion			0.640
Present	8	8	
Absent	11	8	
Necrosis			0.142
Present	6	9	
Absent	13	7	
Ki–67 (%)[a]			0.127
<80	10	4	
≥80	9	11	
B symptoms			0.536
Present	13	13	
Absent	6	3	
LDH[b]			0.358
Normal	5	8	
Elevated	12	10	

[a] Data of Ki-67 were available in 34 patients

[b] Data of LDH were available in 32 patients

*$P \leq 0.05$ was considered statistically significant

The present study revealed that 35/42 (83.3%) of ENKTCL patients and 6/10 (60%) of chronic nasopharyngitis patients had del-LMP1. Some studies suggested that the prevalence of del-LMP1 in ENKTCL might be attributed to the prevalence of this variant in the general population [6, 29], while others reported a significantly higher association of ENKTCL compared to healthy individuals [16, 27, 32, 33]. Chiang et al. [27] found a significantly higher prevalence of del-LMP1 in ENKTCL (91.3%) compared to normal nasal tissue (62.5%). The observation was further supported by Tai et al. [33], who reported that del-LMP1 was detected in all UADT lymphomas (100%) and proposed a predilection for del-LMP1 in UADT lymphomas. Despite the limited number of biopsies, majority ENKTCL patients in our study carried del-LMP1.

Regarding various clinicopathologic characteristics, including gender, age, Ann Arbor stage, B symptoms, LDH, vascular invasion, and necrosis, del-LMP1 was not correlated with ENKTCL. Although del-LMP1 presumably contributes to the ability to transform during ENKTCL tumorigenesis, it may have no actual effect on the clinical characteristics or the clinicopathological manifestations. In the survival analysis, we found that the two ENKTCL patients with wt-LMP1 survived longer than the median survival time of patients with del-LMP1, suggesting a better prognostic trend. This may be due to del-LMP1's ability to activate NF-κB abnormally, making cells resistant to apoptosis and promoting lymphoma development [36]. Alternatively, the loss of part of the genetic structure may weaken the immunogenicity of the LMP1 protein, giving tumor cells a survival advantage under immune surveillance, prolonging and enhancing the tumorigenic effect, and maintaining or promoting the evolution of lymphoma [35]. However, given the limited number of wild-type cases, del-LMP1 should be viewed objectively, and it cannot be regarded simply as a pathogenic factor of ENKTCL, but we cannot completely deny that del-LMP1 may play a role in the occurrence and development of this lymphoma.

Although ENKTCL is considered to exhibit type II latency, LMP1 protein expression was detected in only 21

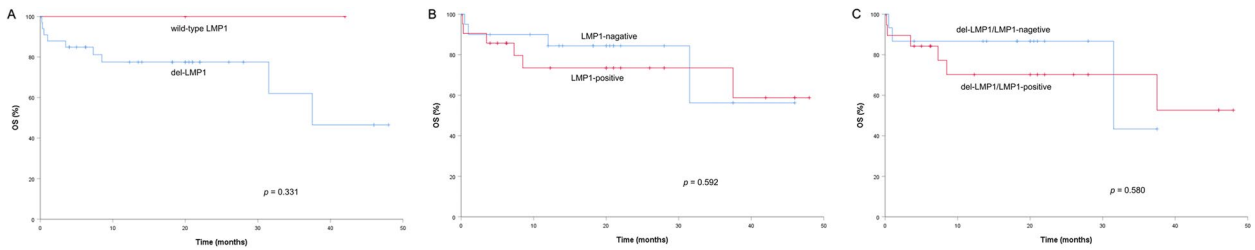

Fig. 5 Overall survival (OS) curves in patients with ENKTCL. **A** Overall survival rate of individuals with and without del-LMP1 in ENKTCL patients ($p = 0.331$). **B** Overall survival rate of individuals with LMP1-positive and -negative in ENKTCL patients ($p = 0.592$). **C** Overall survival rate of individuals with LMP1-positive and-negative in ENKTCL patients with del-LMP1 ($p = 0.580$)

(50%) cases in our study, of which 19 positive specimens carried del-LMP1. While previous studies have demonstrated that LMP1 protein expression was highly correlated with B symptoms [41] and anatomic location [42], our study did not find significant differences. However, our results indicated a trend toward increased LMP1 protein expression in patients with elevated LDH and younger patients (≤60 years old). In particular, LMP1 protein expression was significantly higher in young patients (≤60 years old) than in elderly patients (>60 years old) within the del-LMP1 group ($p=0.004$). This may be the reason why the majority of ENKTCL patients are younger than 60 years old (median age in this study: 55 years), and this finding may indirectly support the hypothesis that LMP1 protein products carrying 30-bp deletion exhibit reduced immunogenicity, thereby conferring a survival advantage and enhancing pathogenicity [35]. Further exploration with larger follow-up samples is necessary to establish the correlation between LMP1 protein carrying the 30-bp deletion and patient age. Unlike previous studies, we observed that LMP1-positive tumor cells were more frequently detected in tissues with coagulative necrosis, potentially due to the cytopathic effect of LMP1-positive tumor cells. Tissue necrosis is a key feature of ENKTCL, but not always observed. This may be due to the fact that the biopsy specimens were usually small or due to variations in sampling locations. Additionally, early-stage disease may not yet involve vascular invasion and destruction, leading to tissue ischemia and extensive necrosis [43]. Knecht et al. [15] speculated in their study of HD cases that the strong expression of LMP1 protein in necrotic areas can cause a reduced amount of protein turnover, which may lead to the cytopathic effect of LMP1. The implication of the association between LMP1-positive expression and coagulative necrosis in ENKTCL requires further investigation. Studies by Jiang et al. [44] and Cao et al. [41] have shown that LMP1 expression was associated with an unfavorable prognosis in ENKTCL. In our study, there was no statistically significant difference in overall survival between the LMP1 protein-positive and negative groups. However, we observed a lower 1-year survival rate in the LMP1 protein-positive group than in the negative group. This may be due to the reduced immunogenicity of the highly mutated LMP1 isolate protein, which escapes immune surveillance and promotes lymphoma progression.

In recent decade, the detection methods of del-LMP1 have significantly advanced. Next generation sequencing (NGS) technology has identified additional variants, including STAT3, JAK3, STAT5B, MSN, BCOR, DDX3X, TP53, and MGA [26]. Despite its advantages, NGS remains relatively expensive and can raise difficult challenges for analysis and interpretation, limiting its application for diagnostic applications. Sanger sequencing, characterized by high sensitivity, convenient operation, and relatively low cost, may be particularly useful in resource-limited countries. In our experiment, we observed that the detection rate of the LMP1 gene was significantly higher ($p=0.048$) than that of the protein. This may be due to varying expression levels of LMP1 protein during different stages of tumor cells differentiation and maturation, or other factors affecting transcription and translation levels, leading to decreased LMP1 protein expression or reduced immunogenicity of mutant LMP1 isolates. It may also be related to technical reasons, such as the difficulty of antigen repair after formalin treatment. The specific reasons need to be further studied and discussed.

Conclusion

Our study demonstrated that del-LMP1 is the most prevalent variant in our population, but it was not associated with overall survival or any investigated clinicopathological characteristics of ENKTCL. Therefore, from the viewpoint of molecular epidemiology, del-LMP1 appears to be the predominant variant than a phenotype-associated polymorphism in ENKTCL. Additionally, we observed that LMP1 protein expression was more common in younger patients with del-LMP1 and in tissues with necrosis. These findings contribute to the understanding of the information on LMP1 characteristics in ENKTCL patients in Wenzhou. Expanding the sample size and continuing follow-up will be essential to fully elucidate the significance of LMP1 in ENKTCL, including its predictive and prognostic value.

Abbreviations

ENKTCL	Extranodal natural killer/T-cell lymphoma
WHO	World Health Organization
NPC	Nasopharyngeal carcinoma
EBV	Epstein-Barr virus
EBER	EBV-encoded small RNA
OS	Overall survival
LDH	Lactate dehydrogenase
LMP1	Latent membrane protein 1
UADT	Upper aerodigestive tract
HD	Hodgkin's disease
PCR	Polymerase chain reaction
IHC	Immunohistochemistry
ISH	In situ hybridization
NGS	Next generation sequencing
FFPE	Formalin-fixed paraffin-embedded
DNA	Deoxyribonucleic acid

Authors' contributions

X.L. designed the entire study. Q.H. designed the primers. X.L., P.L., K.H., X.J., S.C., R.L. and X.W. collected clinical data and performed research. X.L. wrote the manuscript. The authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was approved by the ethics committee in clinical research (ECCR) of the first affiliated hospital of Wenzhou Medical University (No. KY2021-R081). For the ECCR waived this retrospective study informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Pathology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, China. [2]Department of Animal Science, Wenzhou Vocational College of Scienc & Technology, Wenzhou, Zhejiang 325006, China.

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