



Mutational profile and EBV strains of extranodal NK/T-cell lymphoma, nasal type in Latin America

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Received: 9 September 2019 / Revised: 19 October 2019 / Accepted: 22 October 2019 / Published online: 10 December 2019
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Abstract

Extranodal NK/T-cell lymphoma (ENKTL) is an Epstein-Barr virus (EBV) associated lymphoma, prevalent in Asia and Latin America. Studies in Asian cohorts have identified some recurrent gene mutations in ENKTL; however, the mutational landscape of ENKTL in Latin America is unknown. In this study, we investigated the mutational profile and EBV strains of 71 ENKTL cases from Latin America (42 from Mexico, 17 from Peru, and 12 from Argentina) and compared it with Asian cohorts. The mutational analysis was performed by next generation sequencing (NGS) using an Ion AmpliSeq™ custom panel covering for the most frequently mutated genes identified in ENKTL. *STAT3* was the most frequent mutated gene (16 cases; 23%), followed by *MSN* (10 cases; 14%), *BCOR* (9 cases; 13%), *DDX3X* (6 cases; 8%), *TP53* (6 cases; 8%), *MGA* (3 cases; 4%), *JAK3* (2 cases; 3%), and *STAT5B* (1 case; 1%). Mutations in *STAT3*, *BCOR*, and *DDX3X* were nearly mutually exclusive, suggesting different molecular pathways involved in the pathogenesis of ENKTL; whereas mutations in *MGA*, *MSN*, and *TP53* were concomitant with other mutations. Most cases (75%) carried Type A EBV without the 30-bp *LMP1* gene deletion. The overall survival was significantly associated with serum LDH level, Eastern Cooperative Oncology Group (ECOG) performance status, International Prognostic Index (IPI) score, and therapy ($p < 0.05$), but not associated with any mutation, EBV strain or deletion in EBV *LMP1* gene. In conclusion, mutational analysis of ENKTL from Latin America reveals frequent gene mutations leading to activation of the JAK-STAT pathway (25%), mostly *STAT3*. Compared to Asian cohorts, *BCOR*, *DDX3X* and *TP53* mutations were also identified but with different frequencies. None of these mutations were associated with prognosis.

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Supplementary information The online version of this article (<https://doi.org/10.1038/s41379-019-0415-5>) contains supplementary material, which is available to authorized users.

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Introduction

Extranodal NK/T-cell lymphoma, nasal type (ENKTL) is a rare and highly aggressive non-Hodgkin lymphoma. According to the 2016 revised World Health Organization

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(WHO) classification of lymphoma, ENKTL is an extranodal lymphoma of NK- or T-cell lineage, characterized by vascular damage, prominent necrosis, cytotoxic phenotype and association with Epstein-Barr virus (EBV) in 100% of cases [1]. Most cases originate from mature NK cells with expression of the NK-cell marker CD56 and lack of T-cell receptor (TCR) gene rearrangement [2–4]. However, a minority of cases derive from cytotoxic T cells with expression of TCR $\alpha\beta$ or TCR $\gamma\delta$ and clonal TCR gene rearrangement [4, 5]. The etiology and pathogenesis of ENKTL are still unclear, but the common association with EBV suggests its pathogenetic role. EBV infection is thought to be an early event in the pathogenesis of ENKTL, and additional genetic alterations might be crucial to induce lymphomagenesis [6]. EBV exists in a clonal episomal form in the tumor cells and shows a type II latency pattern. Most cases are infected by type A EBV with geographic variation [7], and a 30-base pair (bp) deletion in *LMP1* gene is common in Asian cohorts, associated with decrease in immune recognition and increase in tumorigenicity [8, 9]. ENKTL mostly involves the upper aerodigestive tract, mainly the nasal cavity followed by nasopharynx, paranasal sinuses, and palate. However, about 20% of cases occur in extranasal sites, particularly skin, gastrointestinal tract, soft tissue, and testis [10].

ENKTL is more prevalent in Asians and the indigenous population in Mexico, Central and South America, indicating a strong genetic background [1]. ENKTL accounts for 40% and 13% of all T- or NK-cell lymphoma cases in Mexico and Peru, respectively, in contrast to 4% to 6% in the United States and Europe [11]. Despite multi-drug chemotherapy and/or radiotherapy, the 5-year survival rate is still low (40–60%), especially for extranasal cases [12–15]. However, few cases reveal a relatively indolent and protracted clinical course, associated with low Ki-67 proliferation index [16]. Although our understanding of ENKTL has increased in the last few years, data about the genetic alterations of the disease are limited due to its rarity and the commonly observed extensive tumor necrosis [17]. With the advancement of next generation sequencing (NGS) technology, some studies have demonstrated recurrent mutations, copy number variations, and epigenetic changes, particularly hypermethylation, of various genes in ENKTL, including RNA helicase gene *DDX3X*, members of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway (*JAK3*, *STAT3*, *STAT5B*), tumor suppressor genes (*TP53*, *PRDM1*, *FOXO3*, *HACE1*), oncogenes (*RAS*, *MYC*), epigenetic modifiers (*MLL2*, *ARIDIA*, *EP300*, *ASXL3*), as well as genes regulating cell cycle (*CDKN2A*, *CDKN2B*, *CDKN1A*) and apoptosis (*FAS*) [18–20]. The first genetic alteration described in ENKTL was *TP53* mutation in 24% of the cases in a Mexican cohort, associated with large cell

morphology and advanced stage at presentation [21]. Activating mutations involving JAK-STAT signaling pathway (*JAK3*, *STAT3* and/or *STAT5B*) have been reported in 12.4–35% of ENKTL cases and appear to play a key role in the pathogenesis [18–20, 22, 23]. In addition, *BCOR* and *DDX3X* have been reported as the most frequent recurrent mutations in ENKTL in Japan [22] and China [20], respectively. In addition, several candidate tumor suppressor genes (*PRDM1*, *ATG5*, *AIM1*, *HACE1*, and *FOXO3*) have been identified in the commonly deleted region on chromosome 6q21 in ENKTL. All of these studies have been conducted in Asian populations (China, Korea, and Japan), and a large-scale mutational analysis from Latin America is still lacking. In this study, we performed mutational analysis in 71 ENKTL cases from Latin America by targeted NGS and found that *STAT3* mutation was the most frequent gene alteration. Furthermore, we now demonstrate that type A EBV without the *LMP1* 30-bp deletion is the most common strain in ENKTL in Latin America.

Materials and methods

Lymphoma samples and clinical data

For this study, 135 cases of ENKTL were collected from six reference centers in Latin America, including Instituto Nacional de Cancerología (Mexico City, Mexico), Instituto Nacional de Enfermedades Neoplásicas (Lima, Peru), Hospital Ángel C. Padilla (San Miguel Tucumán, Argentina), Instituto de Oncología Ángel H. Roffo (Buenos Aires, Argentina), Hospital Italiano de Buenos Aires (Buenos Aires, Argentina), and Instituto de Investigaciones Hematológicas (Buenos Aires, Argentina). The medical records were reviewed, including age, sex, previous medical history, disease presentation and extent, staging, B symptoms, International Prognostic Index (IPI) score, Eastern Cooperative Oncology Group (ECOG) performance status, treatment, and follow-up. Overall survival (OS) was calculated from the date of diagnosis to 1-year or 5-year, or the last follow-up. All cases were reviewed by three pathologists (IAM-M, B-JC, and LQ-M.) following the criteria of the 2016 revised WHO classification of lymphoma [1]. This study was performed according to the Declaration of Helsinki and approved by the local Ethics Review Committee of the contributing institutions (780/2016B02).

Immunohistochemistry and EBER in situ hybridization

All cases were immunophenotyped as part of routine diagnosis. Immunohistochemistry for CD56 (MRQ-42,

Medac GmbH, Wedel, Germany), and EBV-encoded small RNA (EBER) in situ hybridization (ISH) were performed on FFPE tissue sections to confirm the diagnosis. In addition, to correlate mutational status and protein overexpression, immunohistochemistry of phospho-STAT3 (Y705, Cell Signaling Technology, Danvers, MA, USA) and p53 (DO-7, Novocastra, Leica Biosystems, Wetzlar, Germany) were performed. The immunostaining and the EBER ISH were performed using an automated stainer (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocol.

DNA isolation

Tumor DNA was extracted from 135 ENKTL cases using the Maxwell 16 FFPE Plus LEV DNA Purification Kit (Promega, Madison, WI, USA) and quantified with the Qubit Fluorometer employing the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. Quality control polymerase chain reaction (PCR) was performed to determine the amplifiable DNA length in different cases [24]. Only 71 cases with at least 200 bp amplifiable DNA were included into the NGS analysis. Sixty-four cases were excluded because of poor DNA quality due to extensive tumor necrosis.

PCR analysis of EBV strain and 30-bp *LMP1* gene deletion

PCR analysis for EBV subtypes A and B was performed, as previously reported [25]. Primers flanking a region of the *EBNA2* gene differing between subtype A and subtype B EBV were used: *EBNA2* F (5' AGGCTGCCACCT-GAGGAT 3') and *EBNA2* R (5' GCCACCTGG-CAGCCCTAAAG 3'). Each reaction was analyzed in duplicates using 20 ng of extracted DNA in 50 μ l volume with positive and negative controls [26]. Cases with expected DNA fragments of 168 bp were interpreted as EBV subtype A, and DNA fragments of 184 bp product length were interpreted as EBV subtype B [9]. PCR reactions to identify the 30-bp *LMP1* gene deletion were carried out using primers flanking the characteristic 30-bp deletion: *LMP1* F (5' CGGAAGAGGTGGAAAACAAA 3') and *LMP1* R (5' GTGGGGTTCGTCATCATCTC 3'), rendering a 161 bp product for wild-type *LMP1* and 131 bp for the deletion variant [25].

Targeted NGS analysis and correlation with immunohistochemistry

The targeted NGS analysis was performed on the Ion Torrent Personal Genome Machine (PGM) (Thermo Fisher

Scientific, South San Francisco, CA, USA). Total 71 ENKTL cases were selected for NGS analysis; the other 64 cases were excluded due to the low DNA quality or high content of necrosis leading to sequencing artefacts. NGS analysis was performed using a designed Ion AmpliSeq™ custom panel covering eight of the most frequently mutated genes found in ENKTL, including *STAT3*, *STAT5B*, *JAK3*, *DDX3X*, *TP53*, *MGA*, *MSN*, and *BCOR* [18, 20, 23]. The custom panel was designed with the Ion AmpliSeq Designer from Thermo Fisher Scientific (version 3.4) and is summarized in Supplemental Table 1.

Genes of interest were amplified with the designed AmpliSeq custom panel primers. Quantitation of the libraries was done using the Library Quantitation Kit on the LightCycler 480 (Roche, Basel, Switzerland) to ensure proper amplification of the target regions. Clonal amplification took place on the Ion OneTouch instrument (Thermo Fisher Scientific, South San Francisco, CA, USA) using Ion Sphere particles that were subsequently enriched on the Ion OneTouch ES and loaded on a semiconductor chip for sequencing on the Ion Torrent PGM. All steps were performed according to the manufacturer's protocols (Thermo Fisher Scientific). The data generated at the end of the run was automatically transmitted to the Ion Torrent server for further processing and analysis with specific algorithms. Variant calling was performed using the Ion Reporter software (Thermo Fisher Scientific); the obtained variants were analyzed in the freely available program Integrative Genomics Viewer (IGV, Broad Institute) [27] and verified in accordance with known single nucleotide polymorphisms database such as Exome Variant Server and Clinically Associated Human Variations of the National Center for Biotechnology Information (NCBI). To confirm mutational signatures, we compared them to the current Catalog of Somatic Mutations in Cancer signatures and previous studies of somatic mutations using freely available online programs. In order to evaluate the impact of missense variants, these were analyzed by the prediction tools sorting intolerant from tolerant (SIFT) [28, 29] and Polymorphism Phenotyping v2 (PolyPhen-2) [30].

Mutations with low variant allele frequencies (VAF < 10%) were validated in a second run on the Ion Torrent using a targeted resequencing approach using the fusion method (Life Technologies, Thermo Fisher Scientific). Design of the primers was carried out with the freely available online program Primer3web (version 4.0.0). The primers were used in order to amplify regions of interest in a standard PCR. The subsequent workflow was similar to the method described above for the Ion Torrent PGM. The VAF of these mutations was correlated with the percentage of viable tumor cells in tissue sections, based on morphology, CD56 immunostaining, and EBER.

Statistical analysis

Statistical analysis was performed using IBM SPSS v.25 (Armonk, New York, USA). Categorical variables were described using frequencies and proportions; numerical variables were reported as either means and standard deviation (\pm SD) or medians and interquartile range, depending on the distribution of the data. Normality of the distribution was assessed by investigating kurtosis, skewness as well as Q–Q plots. Comparison of mutations, EBV strains, and *LMP1* gene deletion in different countries was assessed with bivariate analysis using the Chi-square test or the Fisher's exact test as appropriate to assess the association between each categorical variable and case-control status. *P*-value < 0.05 with two-sided analysis was considered statistically significant. Kaplan–Meier analysis and univariate Cox regression analysis were performed to assess the clinical value of the clinicopathological parameters and mutations on OS; hazard ratio and their 95% confidence intervals (95% CI) were calculated.

Results

Clinical and morphological findings in ENKTL in Latin America

A total of 71 patients were included in this study with good DNA quality suitable for NGS analysis, including 42 patients from Mexico, 17 patients from Peru, and 12 patients from Argentina. The clinical information is summarized in Table 1. The median age was 40 years (range, 14–83 years). The male-to-female ratio was 2:1. Fifty-eight cases (89%) had nasal involvement, while seven cases (11%) presented in extranasal organs without nasal involvement (one stomach, two small intestine, one vulva, one lung, one submandibular soft tissue, and one lymph node with extranodal systemic dissemination). According to the available clinical information, two-thirds of patients had B symptoms and two-thirds of patients had elevated serum LDH level. Twenty patients (39%) had ECOG performance status of 2–5, and 12 patients (24%) had IPI score of 3–5 at presentation. Twenty-six (52%), 10 (20%), and 10 (20%) of the patients received combined chemoradiotherapy, chemotherapy alone, and radiotherapy alone, respectively. Four patients (8%) did not receive therapy. Follow-up data was obtained from 51 patients; 25 patients (49%) died of disease, 18 patients (33%) showed no evidence of disease, and 8 patients (16%) were alive with disease at last follow-up. The median follow-up was 18.27 months (range 0–118.6 months) and the median survival was 61.6 months. The 1- and 5-year survival rate was 57% and 51%, respectively. Morphologically, the cases revealed variable

Table 1 Clinical data and outcome of extranodal NK/T-cell lymphoma patients in Latin America.

| | Total (<i>n</i> = 71) |
|--------------------------------------|------------------------|
| Median age (range) | 40 (14–83) |
| Gender (<i>n</i> = 62) | |
| Male | 40 (65%) |
| Female | 22 (35%) |
| M:F ratio | 2:1 |
| Site of involvement (<i>n</i> = 65) | |
| Nasal | 58 (89%) |
| Extranasal | 7 (11%) |
| B symptoms (<i>n</i> = 51) | 34 (67%) |
| LDH elevation (<i>n</i> = 51) | 34 (67%) |
| ECOG (<i>n</i> = 51) | |
| 0–1 | 31 (61%) |
| 2–5 | 20 (39%) |
| IPI score (<i>n</i> = 49) | |
| 0–2 | 37 (76%) |
| 3–5 | 12 (24%) |
| Therapy (<i>n</i> = 50) | |
| Combined chemoradiotherapy | 26 (52%) |
| Chemotherapy alone | 10 (20%) |
| Radiotherapy alone | 10 (20%) |
| No therapy | 4 (8%) |
| Outcome (<i>n</i> = 51) | |
| DOD | 25 (49%) |
| NED | 18 (33%) |
| AWD | 8 (16%) |
| Median survival in months | 61.6 |
| Overall survival rate | |
| 1-year | 57% |
| 5-year | 51% |

AWD alive with disease, DOD dead of disease, ECOG Eastern Cooperative Oncology Group, IPI International Prognostic Index, LDH lactate dehydrogenase, NED no evidence of disease

degrees of necrosis (0–90% of the tumor tissue), angiocentricity and angiodestruction (Fig. 1a–i). The neoplastic cells were mostly intermediate-sized with irregular nuclear membrane, hyperchromasia, and pale to clear cytoplasm. There were also some cases displaying large cell morphology or small cell appearance mimicking reactive small lymphocytes (Fig. 1c, f, i). All cases were positive for CD56 and EBER ISH (Fig. 1b, e, h).

Identification of recurrent somatic mutations by targeted NGS

All 71 cases were analyzed by NGS. The mean average read depth of the NGS sequence analysis was 4063 (range, 413–6334). All mutations are shown in Fig. 2 (detailed

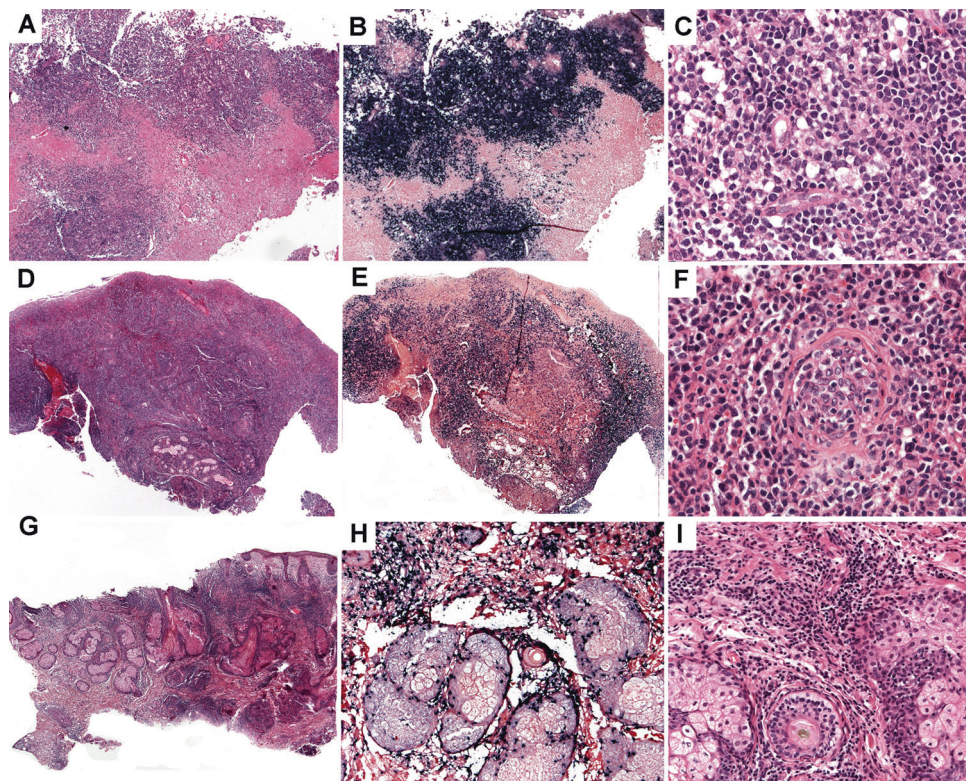
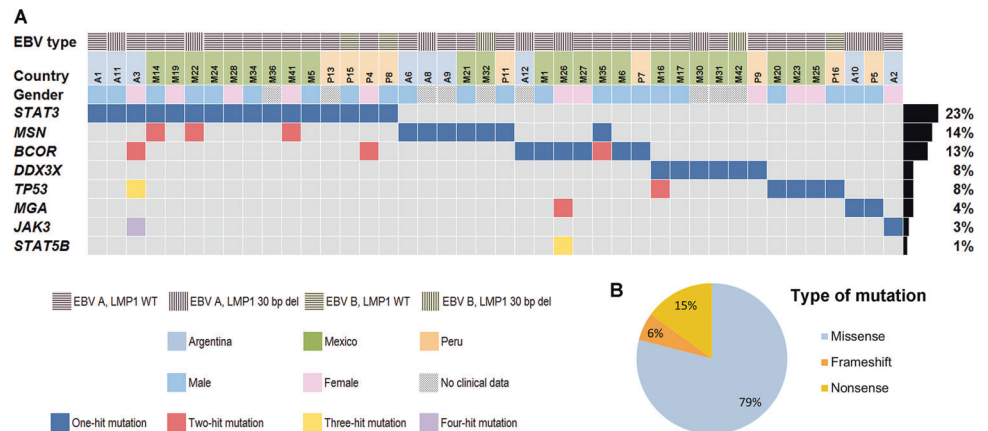


Fig. 1 Morphology of extranodal NK/T-cell lymphoma, nasal type (ENKTL). **a** H&E stain of an ENKTL tumor showing extensive lymphoid infiltration and tumor necrosis. **b** EBER ISH demonstrating that the majority of the tumor cells are EBV positive. **c** Higher magnification reveal large cell morphology with nuclear irregularities and pale to clear cytoplasm. **d** H&E stain of an ENKTL case with ulceration and dense tumor infiltration. **e** The tumor cells are positive

for EBER ISH. **f** Higher magnification reveals angioinvasion by medium-sized lymphoid cells. **g–i** An ENKTL case in the skin. **g** H&E stain reveals a lymphoid infiltrate in the dermis that surrounds the adnexae. **h** The small cells are EBV positive. **i** Note the rather subtle lymphoid infiltrate with bland-looking, small lymphoid cells mimicking a reactive lesion. (**a, d, g**: H&E stain, $\times 25$; **c, f**: H&E stain, $\times 400$; **i**: H&E stain, $\times 200$; **b, e**: EBER, $\times 25$; **h**: EBER, $\times 200$).

Fig. 2 Overview of mutational profile, country distribution and EBV strain of ENKTL in Latin America. **a** Each column of the heat map represents 1 ENKTL case and each line 1 specific analysis. On the right side of the figure, the frequency of the particular result of the analysis is shown. Gene mutations were identified in 42 of 71 cases (59%). **b** Type of mutations identified.



information is given in Supplemental Table 2) Fifty-three mutations were identified in 42 of 71 cases (59%). Twenty-nine cases (41%) were wild type for all eight genes analyzed; 34 cases showed only one mutation (48%); eight cases displayed more than one mutation (11%). Among all mutations, there were 42 missense point mutations (79%), eight nonsense mutations (15%), and three frameshift insertions (6%). Nonsense mutations were found in *MSN*,

BCOR, *MGA* and *DDX3X* genes; frameshift insertions were identified in *MSN* and *MGA*. All mutations found in *STAT3*, *STAT5B*, *JAK3*, and *TP53* were missense mutations. Analysis by SIFT and PolyPhen-2 predicted a damaging effect for all the reported missense mutations (Supplemental Table 2). VAF of the mutations in all genes ranged from 5 to 77% (mean, 33%); the VAFs were correlated with tumor content.

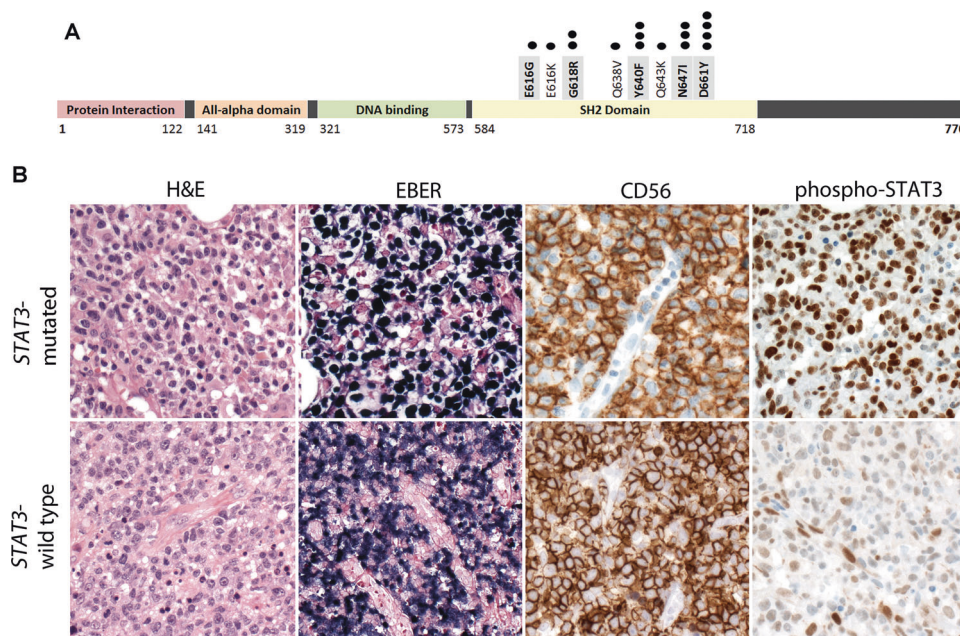


Fig. 3 Distribution of *STAT3* mutation on protein level and protein expression. **a** *STAT3* protein with its different domains. Domains of the protein are represented according to the Uniprot database (www.uniprot.org; UniProtK P40763). Exact positions of each *STAT3* mutation found in 15 ENKTL cases are depicted. All mutations are located within the SH2 domain with hotspots in D661Y, N647I, and Y640F. **b** A *STAT3*-mutated case reveals homogeneous and stronger

staining for phospho-*STAT3*. In contrast, a *STAT3* wild-type case displays heterogeneous and weaker staining of phospho-*STAT3* with stronger staining of endothelial cells as internal control. Note that both cases have EBER and CD56 expression in all tumor cells. (H&E stain, EBER in situ hybridization, CD56 immunostaining, and phospho-*STAT3* immunostains; all x400).

Alterations were most frequently found in *STAT3* gene with 16 mutated cases (23%) and VAF ranging from 6.7% to 69%, followed by *MSN* mutation in ten cases (14%), *BCOR* mutation in 9 cases (13%), *DDX3X* mutations in 6 cases (8%), *TP53* mutations in six cases (8%), *MGA* mutations in three cases (4%), *JAK3* mutations in two cases (3%), and *STAT5B* mutation in one case (1%). In total, 18 cases (25%) had one or more mutations in genes involving the JAK-STAT pathway (*JAK3*, *STAT3*, and/or *STAT5B*). *STAT3* mutations were located within the Src homology (SH2) domain in all 16 mutated cases with hotspots in D661Y, N647I, or Y640F (Fig. 3a). Because *STAT3* mutations are predicted to have constitutively activated *STAT3* protein, six cases (three *STAT3*-mutated cases and three wild-type cases) were analyzed with a p-*STAT3* antibody by immunohistochemistry. *STAT3*-mutated cases showed homogeneous and strong staining of p-*STAT3*, while *STAT3* wild-type cases revealed a heterogeneous, weak staining (Fig. 3b–i), confirming the constitutive expression of p-*STAT3*. *TP53* mutations were observed in the DNA binding domain (Fig. 4a). *TP53*-mutated cases (three cases) showed strong p53 immunostaining in the majority of tumor cells. However, the three *TP53*-wild-type cases showed also heterogeneous positivity (Fig. 4b–h). Only strong, homogeneous expression of p53 in the majority of tumor cells correlated with *TP53* mutation.

Mutations of *STAT3*, *BCOR*, and *DDX3X* were nearly mutually exclusive with only two cases showing concurrent *STAT3* and *BCOR* mutations. In contrast, 40% of *MSN*-mutated cases co-occurred with other mutations. There was no difference in the distribution of the mutations among the three countries (Supplemental Table 3), except for *JAK3* mutations, which were exclusively found in two Argentinian cases.

PCR analysis of EBV strain and *LMP1* gene deletion

The result of EBV strain and *LMP1* gene deletion is summarized in Table 2. Sixty-five cases (92%) revealed type A EBV, while only six cases (8%) were type B EBV (Fig. 5a). Fifteen cases (21%) showed the 30-bp deletion in EBV *LMP1* gene (Fig. 5b). All Peruvian cases showed wild-type *LMP1* gene ($p < 0.05$). In the group of type B EBV, all three Mexican cases showed the 30 bp *LMP1* gene deletion, whereas the other 3 cases in Peru and Argentina were *LMP1* wild type. There was no significant difference in the ratio of Type A and B EBV in the three countries.

Correlation of clinicopathologic factors and overall survival

The statistical analyses of clinicopathologic factors and OS are summarized in Supplemental Table 4. The clinical

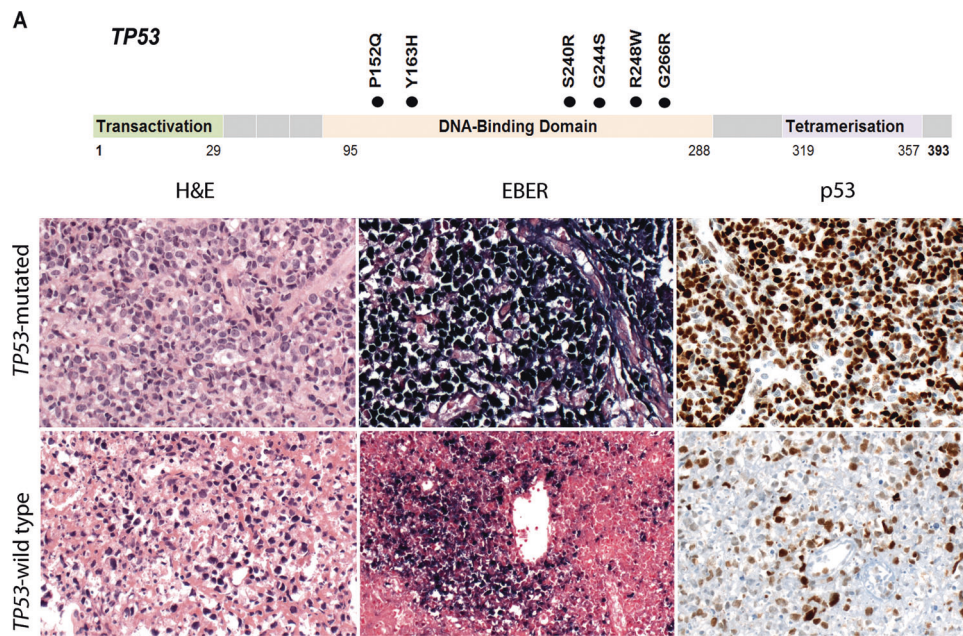


Fig. 4 Distribution of *TP53* mutation on protein level and protein expression. **a** P53 protein with its different domains. Domains of the protein are represented according to the Uniprot database (www.uniprot.org; UniProtK Q6W2J9). Exact positions of each *TP53* mutation found in six ENKTL cases are depicted. **b** A *TP53*-mutated case with large cell morphology, EBER expression, and homogeneous

strong staining of p53 in the majority of tumor cells is shown. In contrast, a *TP53*-wild-type case with tumor necrosis and atypical large tumor cells shows heterogeneous P53 expression in a minority of tumor cells. (H&E stain, EBER in situ hybridization and p53 immunostains; all $\times 400$).

Table 2 EBV strain distribution of extranodal NK/T-cell lymphoma in Latin America.

| | Total (n = 71) | Mexico (n = 42) | Peru (n = 17) | Argentina (n = 12) | P-value* |
|----------------------------|----------------|-----------------|---------------|--------------------|----------|
| EBV strain | | | | | |
| EBV subtype A | 65 (92%) | 39 (93%) | 15 (88%) | 11 (92%) | |
| <i>LMP1</i> wild type | 53 (75%) | 32 (76%) | 15 (88%) | 6 (50%) | <0.05 |
| <i>LMP1</i> 30-bp deletion | 12 (17%) | 7 (17%) | 0 | 5 (42%) | |
| EBV subtype B | 6 (8%) | 3 (7%) | 2 (12%) | 1 (8%) | |
| <i>LMP1</i> wild type | 3 (4%) | 0 | 2 (12%) | 1 (8%) | 1.0 |
| <i>LMP1</i> 30-bp deletion | 3 (4%) | 3 (7%) | 0 | 0 | |

*P-value using Fisher’s exact test with bivariate analysis

bp base pair

information was obtained in 51/71 patients. By univariate analysis, none of these mutations was statistically associated with OS. The OS was correlated with IPI score ($p < 0.001$), ECOG performance status ($p = 0.011$), serum LDH level ($p = 0.008$), and different therapy ($p = 0.014$), but not age, gender, nasal or extranasal involvement, any recurrent genetic mutation, EBV subtype or deletion in *LMP1* gene.

Discussion

In this study, the mutational profile of a large series of ENKTL from Latin America was investigated. This study demonstrated that 25% of Latin American cases carry

activating mutations of the JAK-STAT pathway, mostly *STAT3* mutations. Interestingly, mutations in *STAT3*, *BCOR*, and *DDX3X* were nearly mutually exclusive, indicating their important role in the pathogenesis of ENKTL. In contrast, mutations in *MSN*, *MGA*, and *TP53* were found frequently associated with other mutations, suggesting their potential role in disease progression. There was no significant difference in the mutational profile between the three Latin American countries investigated. Most ENKTL cases (75%) in Latin America were infected with type A EBV without the 30 bp deletion of the EBV *LMP1* gene. Five-year OS rate was rather low (53%), and by univariate analysis, was only associated with IPI score, ECOG performance status, serum LDH level, and different therapies,

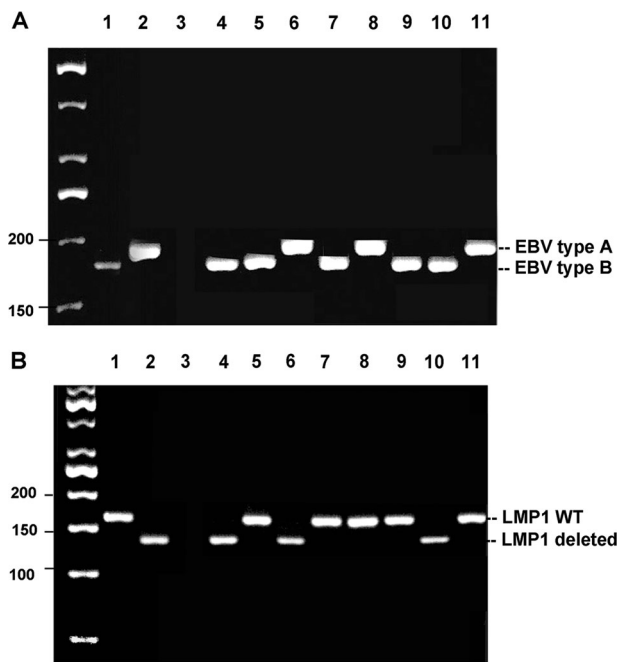


Fig. 5 EBV strain and deletion in *LMP1* gene. PCR analysis of the EBV subtype and *LMP1* deletion of cases from Mexico (lanes 4–6), Peru (lanes 7–8) and Argentina (lanes 9–11) are displayed. **a** Cases of EBV subtype A show expected DNA fragments of 168 bp in lane 4, 5, 7, 9, and 10. Representative cases of EBV subtype B reveal expected DNA fragments of 184 bp in lane 6, 8, and 11. The lanes 1 and 2 are positive controls for EBV subtypes A and B, respectively, whereas the lane 3 is the negative control (no DNA). **b** Representative cases carrying 30-bp deletion of EBV *LMP1* gene show expected DNA fragments of 131 bp in lane 4, 6, and 10. Representative cases of EBV *LMP1* gene wild type (WT) reveal expected DNA fragments of 161 bp in lane 5, 7, 8, 9, and 11. The lane 1 and 2 are positive controls for *LMP1* WT and with 30 bp deletion, respectively, whereas the lane 3 serves as negative control (no DNA).

but not with age, gender, nasal or extranasal involvement, any type or number of mutations, EBV strain or deletion in *LMP1* gene.

Until recently little was known about the genetic landscape of ENKTL. However, in the last years there has been a great interest in this disease, as demonstrated by five large-scale mutational studies in ENKTL from Japan [22], China [19, 20, 31], and Korea [18]. ENKTL is prevalent not only in Asia but also in Latin America; however, similar studies coming from Latin America were missing to date. Probably not surprisingly, our study showed a similar mutational landscape compared to Asian cohorts but with different frequencies (Table 3). *STAT3* was the most frequent recurrent genetic aberration in ENKTL in our study, similar to the Korean study [18]. In contrast, *BCOR* was the most frequent mutation identified in ENKTL in Japan [22] and in an American study including Chinese patients [19]. Another Chinese study described *DDX3X* as the most frequent mutated gene [20]. Although it has been discussed whether these different results might represent diverse genetic backgrounds or geographic distribution, most probably these dissimilarities are due to the various analytic methods (whole-exome sequencing, targeted sequencing, RNA-sequencing, versus Sequenome MassARRAY platform) and materials (tumor tissues versus cell lines) used in these different studies.

Activation of the JAK-STAT signaling pathway has been found as one of the most important events in the pathogenesis of ENKTL [32, 33]. ENKTL and gamma-delta T-cell lymphomas, such as hepatosplenic T-cell lymphoma, primary cutaneous gamma-delta T-cell lymphoma and

Table 3 Comparison of the mutational landscape in Latin America and Asian cohorts

| | Latin America (Current study) | China [27] | Japan [22] | China [20] | China [19] | Korea [18] |
|------------------------------|----------------------------------|--|---------------------|--|---|---|
| Number of cases ^a | 71 | 88 | 25 | 105 | 16 (51 for <i>STAT3</i> and <i>STAT5B</i>) | 30 |
| Technology | Targeted sequencing | 1. WES (5) 2. Sequenome MassARRAY platform (83) | Targeted sequencing | 1. WES (25) 2. Targeted sequencing (80) | 1. WES (1) 2. RNA-seq (15) 3. Sanger sequencing for <i>STAT3</i> and <i>STAT5B</i> (35) | 1. WES (9) 2. RNA-seq (3) 3. Targeted sequencing (21) |
| <i>STAT3</i> | 23% | 3.4% | 16% | 10.5% | 6% (3/51) | 20% |
| <i>MSN</i> | 14% | NA | NA | 8.6% | 0% | 0% |
| <i>BCOR</i> | 13% | 5.7% | 32% | 0% | 20% | 16.7% |
| <i>TP53</i> | 8% | 10.2% | 16% | 13.3% | 13% | 10% |
| <i>DDX3X</i> | 8% | 8% | 12% | 20% | 7% | 0% |
| <i>MGA</i> | 4% | NA | 8% | 8.6% | 0% | 0% |
| <i>JAK3</i> | 3% | NA | 8% | 0% | 0% | 6.7% |
| <i>STAT5B</i> | 1% | 3.4% | 0% | 1.9% | 6% (3/51) | 0% |

^aOnly tumor tissues from ENKTL cases, either fresh or formalin-fixed, paraffin-embedded tissues, are included. Cell lines are excluded

NA not available, WES whole-exome sequencing

monomorphic epitheliotropic intestinal T-cell lymphoma, share similar high frequency of activating mutations within the SH2 domain of *STATs*, suggesting a similar oncogenic mechanism. However, *STAT3* mutations are more frequently identified in ENKTL, while *STAT5B* mutations predominate in other gamma–delta T-cell lymphomas [19]. Activating mutations of genes involved in the JAK-STAT pathway are also present in T-cell prolymphocytic leukemia [34], T-cell large granular lymphocytic leukemia [35], chronic lymphoproliferative disorder of NK cells [36], adult T-cell leukemia/lymphoma [37], Sezary syndrome [38], anaplastic large cell lymphoma [39], primary mediastinal large B-cell lymphoma [40], classic Hodgkin lymphoma [41], and few cases of germinal center B-cell subtype of diffuse large B-cell lymphoma (DLBCL) [40]. The *STAT3* mutations found in this study were all within the SH2 domain, corroborating the data from previous studies [18, 19]. Furthermore, *STAT3*-mutated cases revealed phospho-STAT3 protein, further supporting its constitutive activation in ENKTL. Another gene involved in the JAK-STAT pathway initially reported frequently mutated in ENKTL was *JAK3* with a frequency of 35.4% [23]. However, the frequency of *JAK3* mutations in recent studies has been shown to be relatively low (0–8%) [18, 19, 22, 42], similar to the 3% found in our study. Nevertheless, despite the low *JAK3* mutation frequency, *JAK3* has been reported activated and phosphorylated in 87% of ENKTL [43]. Regardless of the genetic mutations affecting JAK-STAT pathway, it has been suggested that EBV through its LMP1 protein can also activate STAT either by induction of IFN- γ or acting as a protein homolog of the CD40 receptor, that is able to directly activate *JAK3* leading to phospho-Tyr STAT3 [44, 45]. All these data emphasize the importance of activated JAK-STAT pathway in the pathogenesis of ENKTL.

Recurrent *BCOR* mutations are rare in lymphomas and have been reported in 24% of splenic diffuse red pulp small B-cell lymphoma [46] and 16% to 32% of ENKTL [18, 19, 22]. In contrast to previous series, *BCOR* mutations were identified only in 13% in this study. *BCOR* is a tumor suppressor gene, encoding a co-repressor of BCL6, which is an important transcription factor regulating germinal center formation. Through epigenetic and chromatin modifications, *BCOR*, and BCL6 cooperatively silence downstream targets. It has been suggested that the tumor suppressor role of *BCOR* might be blocking NOTCH-dependent transcription [47], and therefore, NOTCH inhibitors may be a potential target therapy in ENKTL [48]. Accordingly, a small-molecule NOTCH inhibitor has been proved to induce growth arrest in NK-cell lines [49]. However, the oncogenic mechanism of *BCOR* dysregulation in lymphomagenesis, especially in ENKTL remains unclear, and more studies and clinical trials are warranted.

Interestingly, we confirmed that mutations in *BCOR* and *DDX3X* genes are mutually exclusive [22]. *DDX3X* is an RNA helicase gene that similar to *BCOR* is considered to function as a tumor suppressor gene [20]. *DDX3X* mutations have been found in 4–20% of ENKTL [19, 20, 22]. Although *DDX3X* mutations have been reported to convey worse prognosis in ENKTL [20], we did not confirm this finding, probably due to the low *DDX3X* mutation rate found in our study (8%). Further studies are needed to elucidate the role and prognosis of *DDX3X* mutations in the pathogenesis of ENKTL. *MSN* mutations were present in ten cases (14%), higher than previously published [18–20]. Forty percent of the *MSN*-mutated cases revealed concurrent *STAT3* or *BCOR* mutations. Interestingly, *BCOR*, *DDX3X* and *MSN* are genes located in the X chromosome. Their loss-of-function mutations might partially explain the male predominance in ENKTL cases.

In a recent whole-exome sequencing study in peripheral mature T and NK-cell lymphomas, *TP53* mutations were found in 60% of intestinal T-cell lymphoma, 22.2% of peripheral T-cell lymphoma, NOS, and 21.7% of ENKTL [50]. Accordingly, *TP53* mutations were associated with extranasal or lymph node involvement, advanced stage, and higher IPI score (>2) [50]. In the present study, *TP53* mutations were found in a relatively low number of cases (6 cases; 8%). Surprisingly, there was no significant difference in OS between *TP53*-mutated and wild-type cases, probably due to the low number of *TP53*-mutated cases in this study.

All together, this study represents the first effort to investigate the mutational landscape of ENKTL in Latin American populations although with a limited gene panel. In order to fully understand the genetic landscape in this population further molecular analysis to investigate copy number variations and chromosomal alterations are warranted.

Another interesting aspect of the study was to investigate the prevalent EBV strain in ENKTL in Latin American cases. Patients with ENKTL in Asia have been reported to have high prevalence of EBV subtypes with the *LMP1* 30 bp deletion, which has higher transforming capacity [8]. In our study, we found that most ENKTL patients in Latin America were infected with type A EBV with wild-type *LMP1* gene. Interestingly, the three cases with type B EBV with the *LMP1* 30 bp deletion were identified in Mexican patients. This result corroborates our previous findings in CHL [9] and DLBCL in Mexican population [26], where we reported a high frequency of type B EBV with *LMP1* 30 bp deletion. In contrast, type B EBV with the *LMP1* 30 bp deletion was not identified in cases from Argentina and Peru. The different EBV strains and *LMP1* gene prevalent in Asian and Latin American cohorts most probably reflect geographic variation of EBV strains types rather than differences in the pathogenesis of the disease or the immune status of the host.

In conclusion, our data extend previous studies and provide further evidence that there are distinct molecular pathways driving ENKTL. We identified the activation of the JAK-STAT pathway, mainly activating *STAT3* mutations, as the most important event in the pathogenesis of ENKTL in Latin American patients. Mutations in *BCOR* and *DDX3X* seem also to be involved in the pathogenesis of ENKTL but were less prevalent than in published Asian cohorts. Nevertheless, no significant difference in OS was found associated to these mutations.

Acknowledgements The authors would like to thank Claudia Hermann and Esther Kohler for their excellent technical assistance. IAMM is supported by funding from the European Union's Horizon 2020 research and innovative Programme under the Marie Skłodowska-Curie grant agreement No 675712.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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