Clonal sequence tracking reveals *TET2*-mutated extranodal NK/T-cell lymphoma disseminated independent of Epstein Barr virus

Extranodal NK/T-cell lymphoma (ENKTL), nasal type, is an aggressive Epstein Barr virus (EBV)-associated lymphoproliferative malignancy with either cytotoxic T- or NK-cell phenotypes. The role of EBV in the mechanism of lymphoma development has been well-recognized whereas the absence of EBV usually negates the diagnosis.[1] In this intriguing case, however, by tracking the common somatic mutations, *TET2* and *DNMT3A*, we showed that the cutaneous dissemination of the nasal type ENKTL was not associated with EBV. This observation might reshape the understanding of the timing of EBV-involving lymphomagenesis.

An otherwise healthy 67-year-old man presented with a nasal tumor, a dark plaque with an erythematous margin on his left cheek, and progressive dissemination of papules on the abdominal skin. Biopsies of both the nasal tumor and facial plaque were taken at the same time. The nasal tumor showed dense intermediate to large-sized lymphoid cell infiltration with coarse chromatin and brisk mitosis. Angiocentricity, necrosis, and pseudoepitheliomatous hyperplasia of nasal epithelium were noted (Figure 1A, B). By immunohistochemistry, the lymphoid cells showed cytotoxic T-cell or NK-cell phenotypes that were negative for CD20, CD4, CD56 and positive for CD3, CD2, CD5(focal), CD8, TIA1, granzyme B (Figure 1C). The tumor cells were diffusely positive for EBV by EBV-encoded small RNA1 (EBER1) *in situ* hybridization and EBV latent membrane protein 1 (LMP1) (Figure 1D). Additional immunostaining revealed they were negative for CD30 and βF1 while positive for T-cell receptor γ (TCRγ) (Figure 1E).

The skin papules showed the same microscopic and immunohistochemical phenotypes as the nasal counterpart (Figure 2A-C), mostly in the dermis without epidermotropism. Surprisingly, the tumor cells in the skin were negative for EBV by EBER1 *in situ* hybridization (Figure 2D) and LMP1. Poor RNA quality was excluded due to the good staining of the RNA *in situ* hybridization control. Additional immunostaining revealed that the tumor cells were also negative for CD30, βF1, and TCRγ (Figure 2E).

To assess the lymphoma clonal evolution, we employed a next generation sequencing based TCR gene rearrangement assay (LymphoTrack® TRG – MiSeq, Invivoscribe, Inc. San Diego, CA, USA) analyzing the formalin-fixed paraffin embedded samples of both tumor sites. Monoclonality was detected in the nasal and skin tumor. The nasal tumor was predominantly formed of a single clone of lymphoid cells (TCRγ: Vg2-Jg1/2; Vg4-Jg1/2 with 35-46% of total reads) whereas the major cutaneous clone (TCRγ: Vg3-Jg1/2; Vg8-Jg1/2 with 34-36% of total reads) constituted approximately 6% of the nasal tumor (Figures 1I, 2I). The major clone in the nasal tumor had an in-frame rearrangement; whereas the major cutaneous clone contained only out-of-frame

Table 1. Clonality analysis by LymphoTrack® TRG.

Tumor site	Clone rank	V-J alignment	of total reads	Sequence	In-frame
Nasal	1	Vg4-Jg1/2	45.65%	GGAATCAGCCCAGGGAAGTATGATACTTACGGAA GCACAAGGAAGAACTTGAGAATG-ATACTGCGAAATCTTATT GAAAATGACTCTGGAGTCTATTAC-TGTGCCACCT GGGATG-GACCAAAATTATTATAAGAAACTCTTTGGCAGTG	No
Nasal	2	Vg2-Jg1/2	35.16%	GGAGTCAGTCCAGGGAAGTATTATACTTACGCAAGCA CAAGGAACAACTTGAGATT-GATACTGCAAAATCTAATT GAAAATGACTCTGG-GGTCTATTACTGTGCCACC TGGG-ACAGGGGGAATTATTATAAGAAACTCTTTGGCAGTG	Yes
Nasal	3	Vg8-Jg1/2	3.33%	GGAATCAGTCGAGAAAAGTATCATACTTATGCAAG CACAGGGAAGAGCCTTAAATT-TATACTGGAAAATCTAATT GAACGTGACTCTGGGGTCTATTACTGTGCCA CCTTATAA-GAAACTCTTTGGCAGTG	No
Nasal	4	Vg3-Jg1/2	3.28%	AGAATCAGTAGAGGAAAGTATTTTACTTATGCAAGCATGAGGA GGAGCTGGAAAT-TGATACTGCAAAATTAATTGAAAATGATTCT CGGATCTATTACTGTGCCACCTGGGA-CCCTAAGTGGGGATTATTAT AAG-AAACTCTTTGGCAGTG	No
Skin	1	Vg3-Jg1/2	35.73%	AGAATCAGTAGAGGAAAGTATTTTACTTATG CAAGCATGAGGAGGAGCTGGAAAT-TGATACTGC AAAATCTAATTGAAAATGATTCTGGATCTATTACT GTGCCACCTGGGA-CCCTAAGTGGGGATTATTATAAG-AAACTCTTTGGCAGTG	No
Skin	2	Vg8-Jg1/2	34.17%	GGAATCAGTCGAGAAAAGTATCATACTTATGCAAGCA CAGGGAAGAGCCTTAAATT-TATACTGGAAAATCTAAT TGAACGTGACTCTGGGGTCTATTACTGTGCCA CCTTATAA-GAAACTCTTTGGCAGTG	No

The table includes the top four clones of the nasal tumor and the top two clones of the skin tumor.

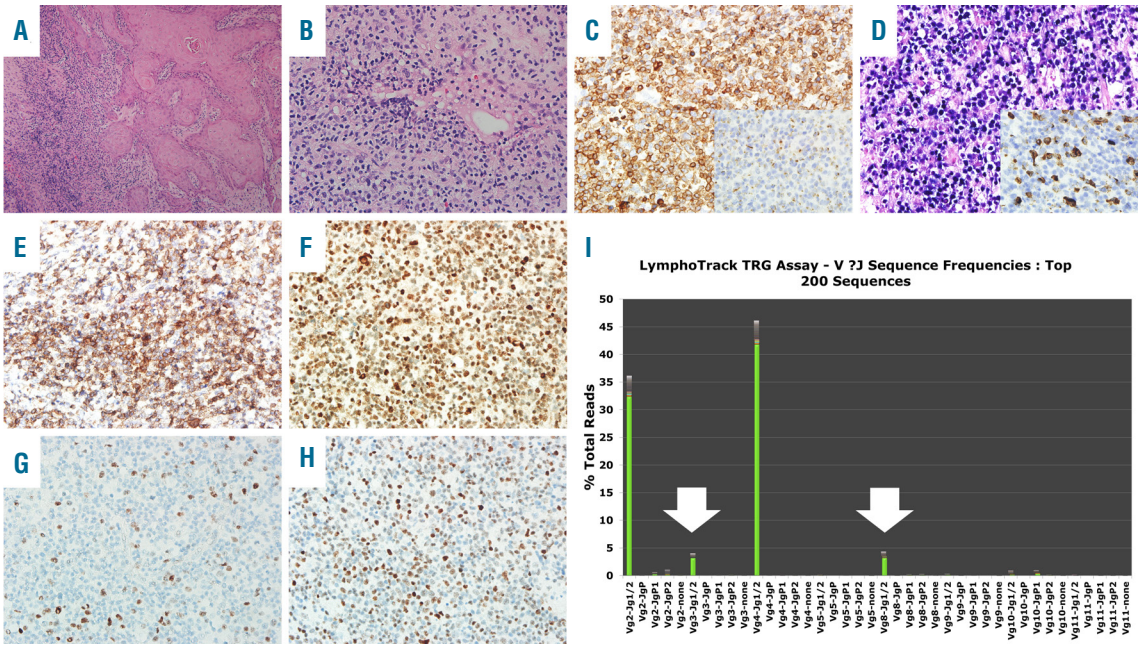

Figure 1. Pathology and TCR analysis of the nasal tumor. All immunostains and chromogenic *in situ* hybridization were performed by automated immunostainer (BENCHMARK® XT, Ventana Medical System, Tucson, AZ, USA). (A) foci of squamous pseudoepitheliomatous hyperplasia mimicking squamous cell carcinoma (X100, H&E). (B) medium to large mature lymphoid cells with angiocentricity (X400, H&E). (C) tumor cells positive for CD3 (X400, CD3) (insert: tumor cells positive for granzyme B, X400). (D) tumor cells diffusely positive for EBV (X400; EBER1 *in situ* hybridization) (insert: tumor cells focally positive for LMP1, X400). (E) tumor cells positive for TCRγ (X400; TCRγ). (F) tumor cells overexpressing phosphorylated STAT3 (X400, pSTAT3). (G) tumor cells slightly overexpressing c-Myc (X400, c-Myc). (H) tumor cells variable overexpression of p53 (X400, p53). (I) V-J sequence frequency graph for the top 200 sequence. The major clone is Vg2-Jg1/2 and Vg4-Jg1/2. The minor clone (marked by a white arrow) is Vg3-Jg1/2 and Vg8-Jg1/2.

rearrangement by IGBLAST® analysis (Table 1). The sequence analysis was consistent with the TCRγ expression in the nasal tumor rather than in the cutaneous tumor. Interestingly, by targeted next generation sequencing (TruSight Myeloid panel, MiSeq), both tumor sites contained a biallelic *TET2* loss-of-function mutation (N704KfsX8 and N275IfsX18; in the nasal tumor the variant allele frequencies (VAFs) were 45.26% and 47.56%; whereas the VAFs in the skin lesion were 38.27% and 38.65%) and a *DNMT3A* mutation (G707D and D702N; in the nasal tumor the VAFs were 44.45% and 47.28%; whereas the VAFs in the skin lesion were 38.81% and 41.42%).

In order to investigate a possible alternative oncogenic pathway in the EBV-negative clone, we tested the oncogenic pathway commonly found in ENKTL, including the JAK-STAT pathway, PRC2 pathway, c-Myc, and p53. Both the nasal and skin tumor expressed phosphorylated STAT3 analysed by immunohistochemistry (Figures 1F, 2F). However, no hotspot mutation in the *STAT3* exon 19-22 and *STAT5B* exon 14-18 was found by polymerase chain reaction (PCR) and Sanger sequencing using the method provided by Küçük *et al.* (2014).[2] Both tumors expressed the PRC2 complex protein, including EZH2 and H3K27me3 and a mild upregulation of c-Myc was found (Figures 1G, 2G). However, no *MYC* rearrangement or copy number changes could be detected by fluorescence *in situ* hybridization (Abbott, Vysis dual-color break-apart probe). Of note, the nasal tumor had variably increased expression of oncoprotein p53 whereas the skin tumor didn't (Figures 1H, 2H). No PD-L1 expression was noted in either tumors, including the tumor cells and adjacent immune cells.

Clinically, the FDG-Positron Emission Tomography scans showed lymphoma involvement in the paranasal sinus, nasopharynx, skin, and lymph nodes in bilateral neck, axilla, and groins. EBV *BLLF1* quantitative PCR reported a serum viral load of 6,309 IU/ml (Abbott RealTime EBV assay). The patient underwent multiple chemotherapies including LOD, SMILE, prophylactic intrathecal chemotherapy, AspaMetDex, daratumumab, VIPD, salvage GELOX, nivolumab, and gemcitabine, sequentially. No durable response was established, and he finally died of the disease one year after diagnosis.

This case of nasal-type ENKTL manifested as one EBV-positive clone in the nasal tumor and one EBV-negative clone in the skin. We postulate that this case was a single disease rather than a synchronous disease at different sites on the basis of the same *TET2* and *DNMT3A* mutation along with a common *TRG* clone. The clone-tracking study in this case raises the possibility that EBV might be dispensable for ENKTL clonal evolution or dissemination, and other genetic mechanisms such as a loss-of-function *TET2* mutation could underpin the various clinical ramifications of this disease. Similar observations about the dispensability of EBV in establishing the diagnosis of aggressive NK-cell leukemia were debated recently.[3-4]

This finding might refine the understanding of how EBV affects the development or dissemination of ENKTL. It has been recognized that EBV is involved in the early development of ENKTL because of its presence in virtually all tumor cells.[5] On the other hand, detection of EBV only in a subset of cells usually suggests a secondary

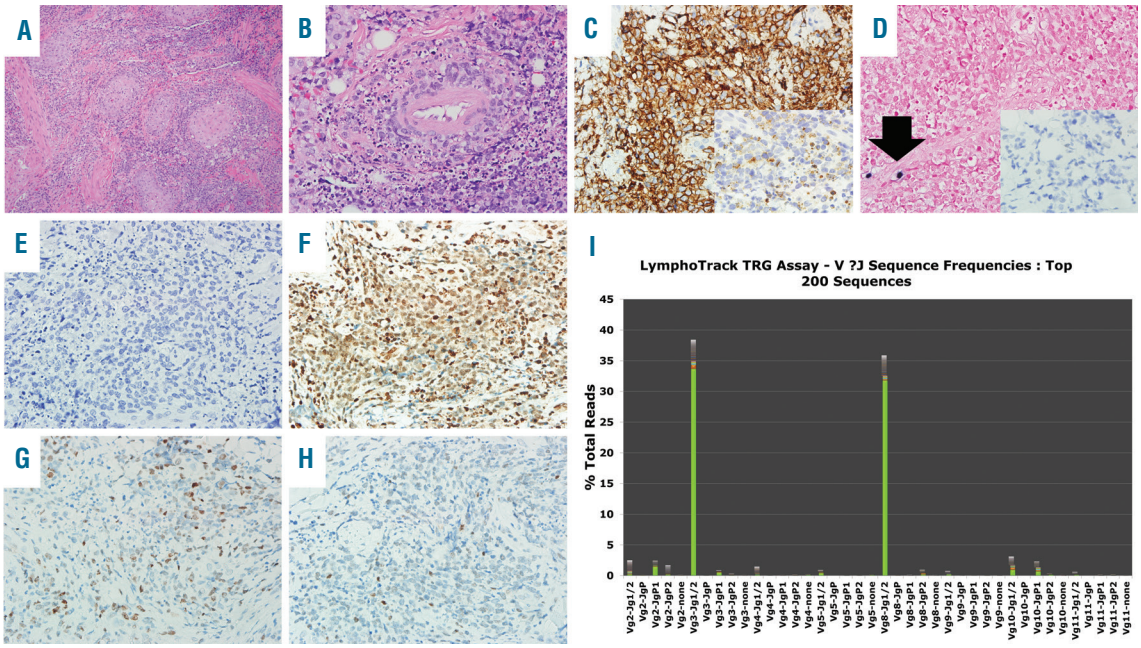

Figure 2. Pathology and TCR analysis of skin tumors. The immunostains and chromogenic *in situ* hybridization were performed by automated immunostainer (BENCHMARK® XT, Ventana Medical System, Tucson, AZ, USA). (A) foci of squamous hyperplasia (X100, H&E). (B) medium to large mature lymphoid cells with angiocentricity (X400, H&E). (C) tumor cells positive for CD3 (X400, CD3) (insert: tumor cells positive for granzyme B, X400). (D) tumor cells negative for EBV (X400; EBER1 *in situ* hybridization). Black arrow: internal controls of EBV on a few small lymphoid cells (insert: tumor cells negative for LMP1, X400). (E) tumor cells negative for TCRγ (X400; TCRγ). (F) tumor cells overexpressing phosphorylated STAT3 (X400, pSTAT3). (G) tumor cells slightly overexpressing c-Myc (X400, c-Myc). (H) tumor cells without p53 deregulation (X400, p53). (I) V-J sequence frequency graph for the top 200 sequence. The major clone is Vg3-Jg1/2 and Vg8-Jg1/2 identical to the minor clone in the nasal tumor.

infection of EBV in a well-developed EBV-negative low-grade lymphoma.[6-7] In this case presented, the cutaneous EBV-negative clone at diagnosis implies that there might be alternative oncogenic mechanisms other than EBV infection or a late episomal loss of the EBV genome during proliferation (so-called "hit-and-run" hypothesis).[8] As the disseminated clone was negative for EBV, we postulate this is mirrored by the relative low serum EBV viral load in our case, in contrast to the serum load one or two orders of magnitude higher in a usual stage IV ENKTL. It is still an open question why an EBV-negative clone manifested more aggressively.

A similar observation of an EBER-negative dissemination of nasal ENKTL was reported by Teo *et al.*[9] Our report, detailed with clonality tracking, mutation analysis, correlation with the serum EBV viral load and surveillance of possible alternative oncogenic pathways, adds another layer of evidence to this special clinicopathological features. It would be interesting to identify possible alternative oncogenic pathways in the EBV-negative clones. It is known that the JAK-STAT pathway, PRC2 pathway, p53, and c-Myc are commonly involved in ENKTL.[2,10-11] Previous two reports of EBV-negative aggressive NK-cell leukemia found similar oncogenic pathways in the usual EBV-positive ones. Interestingly, the STAT3 activating mutation seems to be frequent (40%, in two of the five cases tested).[3-4] This mutation is only present in about 6% of EBV-positive ENKTL.[2] In this case, both clones had activated JAK-STAT, PRC2 pathway, and c-Myc upregulation supported by immunostaining. Of note, the EBV-positive clone overexpressed p53 as expected, while the EBV-negative clone showed no p53 deregulation, supporting alternative oncogenic pathways. Recently, frequent PD-L1 expression has been found in most EBV-driven malignancies, including ENKTL.[12] PD-L1 expression was also found in EBV-negative aggressive NK/T-cell leukemia.[4] These findings have therapeutic implications since tumors expressing PD-L1 show an overall good response to anti-PD1 therapy. However, in our case, both clones were negative for PD-L1, which was consistent with the poor clinical response to nivolumab.

Additionally, we identified the same *TET2* loss-of-function mutations in two different clones of ENKTL. This might suggest that *TET2* mutations occur before *TRG* rearrangement and probably EBV infection or episomal loss of EBER; furthermore, stem cells or lymphoid precursors bearing these mutants might be subjected to a risk of developing ENKTL. There is growing evidence for the role played by *TET2*-mediated epigenetic control of transcription in T-cell lymphoma, mostly in angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma with a follicular helper T phenotype.[13-14] Although uncommon in ENKTL, recognizing these mutations might implicate a possible treatment choice *via* targeting the demethylating oncogenic mechanism of the *TET2* mutation.[15]

There were some limitations in this report. First, it would be more informative, if the presence or not of EBER was found in the minor clone in the nasal tumor (the same clone as the skin tumor). However, it is impossible to separately analyze the major and minor clone in the nasal tumor since the minor clones are too few and did not aggregate apparently. Second, although we found

that p53 was not deregulated in the cutaneous EBV-negative clone, we did not discover a novel oncogenic pathway in the EBV-negative clone. Finally, we performed gene mutation screening by next generation sequencing using a myeloid panel rather than a lymphoid panel. The choice of the myeloid panel was made to discover possible new mutations in this special case. However, this precluded the surveillance of commonly mutated genes in lymphoma.

In summary, we reported a case of biclonal ENKTL with one nasal EBV-positive clone and one cutaneous EBV-negative clone. Deregulation of p53 was not seen in the latter, suggesting an alternative oncogenic pathway. The clonal sequence tracking and the identification of the same *TET2* loss-of-function mutation not only challenges the indispensability of EBV for diagnosing ENKTL, but also repositions the role of EBV in ENKTL development.

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