Hepatosplenic T-cell lymphoma displays an original oyster-shell cytological pattern and a genomic profile distinct from that of γδ T-cell large granular lymphocytic leukemia

Hepatosplenic T-cell lymphoma (HSTCL) is a highly aggressive T-cell neoplasm that arises from the proliferation of gamma/delta T cells (γδT) infiltrating the liver, spleen, and bone marrow sinusoids.[1] It commonly emerges in individuals with chronic immune suppression, predominantly affecting young adults who manifest hepatosplenomegaly, cytopenias, and systemic symptoms.[2] In contrast, large granular lymphocytic leukemia (LGLL) typically manifests as an indolent proliferation of cytotoxic alpha/beta T cells (αβT), primarily afflicting older adults with neutropenia and concurrent autoimmune disorders.[3] Nevertheless, splenomegaly is frequently observed in LGLL, and leukemic cells can carry a γδT-cell receptor (TCR), complicating the differentiation from HSTCL.[4,5] This study, approved by our institution's ethics committee (number 23.34), aims to establish novel diagnostic criteria for distinguishing HSTCL from γδT-LGLL. Herein, we report a distinctive oyster-shell morphology and identify stereotyped VD1-JD1 CDR3 sequences in HSTCL cells.

The morphological description of HSTCL has focused primarily on histological aspects of splenic or hepatic biopsies, with minimal emphasis on cytological features.[6] We centrally reviewed 23 bone marrow aspirate smears, ten blood smears and two biopsy touch preparations (spleen and liver). Bone marrow was involved in all HSTCL cases and blood samples were infiltrated in 42% of cases. The tumor burden in bone marrow ranged from 5% to 73% and varied from sparse neoplastic cells to a diffuse involvement with large pseudometastatic aggregates. Hemophagocytosis was observed in six patients (25%). Atypical lymphoid cells were monotonous, medium-sized, with irregular nuclei displaying fine chromatin and prominent nucleoli. Their cytoplasm had a jagged outline, peripheral basophilic enhancement, and frequent cytoplasmic projections, giving the tumoral cells a distinctive oyster-shell pattern (Figure 1A-C). These cells were predominant within the neoplastic infiltrate and were observed in all the blood and bone marrow samples from HSTCL cases. In touch preparations, tumoral cells often presented with rounder blastic nuclei and reduced cytoplasm, consistent with previous descriptions (Figure 1D).[6] Conversely, LGLL cells had a round nucleus with dense chromatin and pale cytoplasm with azurophilic granules (Figure 1E). Thus, the distinctive oyster-shell morphology observed in both γδT and αβT HSTCL subtypes may provide a new criterion for HSTCL diagnostics (Figure 1F, G). This characteristic could guide additional investigations early in this challenging diagnosis and potentially avoid more invasive procedures. The phenotypic and oncogenic characteristics of the two groups align (Table 1) with previously published data (*Online Supplementary Figure S1*).[2,4] All HSTCL cases were positive for the pan-T-cell markers CD3 and CD2. CD7 was positive in 14 cases (93% of tested cases). Most HSTCL cases were double negative for both CD4 and CD8 (60%). As could be expected, 18 cases (90%) exhibited a γδ-TCR while two cases expressed an αβ-TCR (10%). Several markers might be useful in distinguishing HSTCL from LGLL. A complete lack of CD5 was observed in 17 HSTCL samples (15% of positive cases), whereas LGLL displayed at least weak positivity for CD5 in 81% of cases (P<0.001). CD56 was positive in 13 HSTCL samples (87%), while only two LGLL cases were positive (13%) (P<0.001). Conversely, 14 cases of LGLL expressed CD57 (93%), while CD57 expression was detected in three of the five HSTCL cases tested. Lastly, in four bone marrow biopsies, HSTCL were positive for TIA-1 and negative for granzyme B staining, while this marker is typically expressed in cases of LGLL.[3]

Cytogenetic analysis was available for 14 patients with HSTCL. Isochromosome 7q was detected in ten cases (71%) and trisomy 8 in seven cases (50%). Four patients (29%) had a complex karyotype. Subsequently, we conducted a comparative mutational analysis of 21 HSTCL samples and 16 γδT-LGLL using targeted high-throughput sequencing (*Online Supplementary Table S1*). In the HSTCL group, 17 patients (81%) had at least one somatic mutation. Overall, we identified 30 clinically relevant variants in 13 different genes. The most frequent event was a mutation in the SH2 domain of *STAT5B*, detected in eight patients (38%), with five of them carrying the N642H hotspot. *DNMT3A* was mutated in four cases (19%), *TET2* in three patients (14%), and *EZH2*, *TP53* and *SETD2* in two patients (10%). Among γδT-LGLL, 14 (88%) had at least one significant mutation. The *STAT3* gene was mutated in 12 patients (75%). A *DNMT3A* mutation was found in three cases (19%), and a *TET2* mutation in two cases (13%). Finally, *TNFAIP3, STAT5B*, and *IDH2* mutations were detected in one patient. Consistent with previous studies, the presence of isochromosome 7q remains the most frequent cytogenetic aberration in HSTCL, and it is retained as a diagnostic criterion in the latest revision of the World Health Organi-

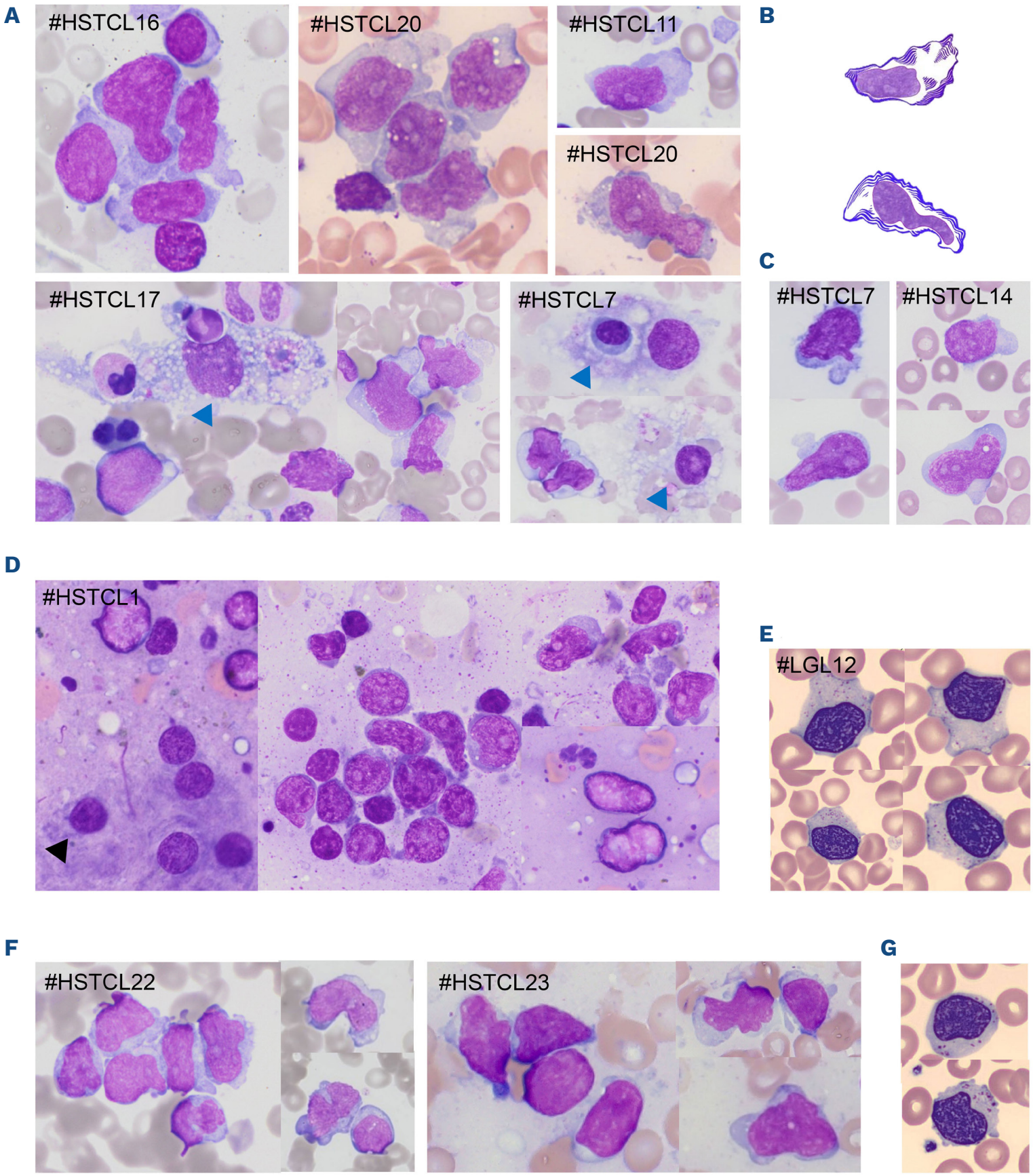

Figure 1. Comparative cytological analysis of hepatosplenic T-cell lymphoma and T-cell large granular lymphocytic leukemia. May-Grünwald-Giemsa staining, magnification x600. (A) Bone marrow aspirate smears from five representative cases of γδ hepatosplenic T-cell lymphoma (HSTCL). Blue arrows indicate images of hemophagocytosis. (B) Schematic illustration depicting the oyster shell pattern observed in HSTCL cases. (C) Tumor cells on blood smears from two γδ-HSTCL cases. (D) Liver biopsy touch preparation from one γδ-HSTCL case. The black arrow points to a Küpffer cell. (E) Large granular lymphocytes (LGL) on blood smears (CellaVision images) from one γδT-LGL leukemia case. (F) Bone marrow aspirate smears from two αβ-HSTCL cases. (G) Blood smear (CellaVision images) from one case of αβT-LGL leukemia.

zation classification.[1,7] The detection of *STAT5B* mutations can provide an additional molecular marker for HSTCL, in contrast to *STAT3* alterations which constitute the molecular hallmark of LGLL.[8] Mutations in genes related to DNA methylation (*TET2* and *DNMT3A*) were identified in both HSTCL and LGLL samples and have been commonly reported in the context of clonal hematopoiesis, making them unsuitable as specific molecular markers for HSTCL. Conversely, *SETD2* appeared to be specifically associated with HSTCL, being present with a frequency of 10% in the cases in our cohort and in up to 25% in previous studies.[9,10] Lastly, exome sequencing provided a more comprehen-

Table 1. Characteristics of patients with hepatosplenic T-cell lymphoma and γδT-cell large granular lymphocytic leukemia.

	HSTCL, N=24	γδT-LGLL, N=16	*P*
Age at diagnosis in years, median (range)	45 (11-83)	63 (31-82)	0.002
Sex ratio, male:female	5	2.5	0.596
Medical history, N (%)			
Inflammatory bowel disease	5 (21)	0 (0)	0.071
Solid organ transplant	2 (8)	1 (14)	0.508
Other malignancies	2 (8)	1 (14)	1.000
Clinical presentation, N (%)			
Splenomegaly	23 (96)	1 (14)	<0.001
Hepatomegaly	16 (67)	0 (0)	<0.001
Lymphadenopathy	0 (0)	0 (0)	1.000
Anemia	19 (79)	2 (29)	0.022
Thrombocytopenia	19 (79)	1 (14)	0 .004
Neutropenia	12 (50)	5 (71)	0.412
Immunophenotypic features, N (%)			
CD3+	20 (100)	16 (100)	1.000
CD4+	1 (5)	0 (0)	1.000
CD8+	7 (35)	9 (56)	0.313
CD5+	3 (15)	13 (81)	<0.001
CD16+	9 (75)	14 (93)	0.153
CD56+	13 (87)	2 (13)	<0.001
CD57+	3 (60)	14 (93)	0.155
Oncogenic profile, N (%)			
Isochromosome 7	10 (71)	NA	NA
Trisomy 8	5 (50)	NA	NA
STAT5B mutations	8 (38)	1(7)	0.050
STAT3 mutations	1 (5)	12 (80)	<0.001
Treatment, N (%)			
Chemotherapy only	10 (42)	7 (44)	NA
Autologous or allogeneic transplant	8 (33)	0 (0)	NA
Untreated	3 (12)	9 (56)	NA
Follow-up			
Median survival in months	11.1	Not reached	NA
Alive at 12 months, N (%)	8 (38)	7 (100)	NA

Continuous variables are reported as the median (range) and discrete variable as a number (percentage among evaluable cases). HSTCL: hepatosplenic T-cell lymphoma; LGLL: large granular lymphocytic leukemia; NA: not available.

sive view of the genomic landscape of HSTCL, revealing alterations in other genes involved in chromatin modification such as *INO8*, *SMARCA2*, and *ARID1B*, as well as in other signaling pathways (e.g., PIK3CD or KRAS), offering potential therapeutic targets for this rare disease.[10,11]

The originality of our study stems from the analysis of TCR rearrangement specificity in a sizable cohort of HSTCL patients using high-throughput sequencing. Recently, Teramo *et al.* investigated the TCR repertoire profile in LGLL, revealing stereotyped *TRG* rearrangements associated with clinical features.[12] Our study provides complementary results on the immunogenetic profile of HSTCL, highlighting

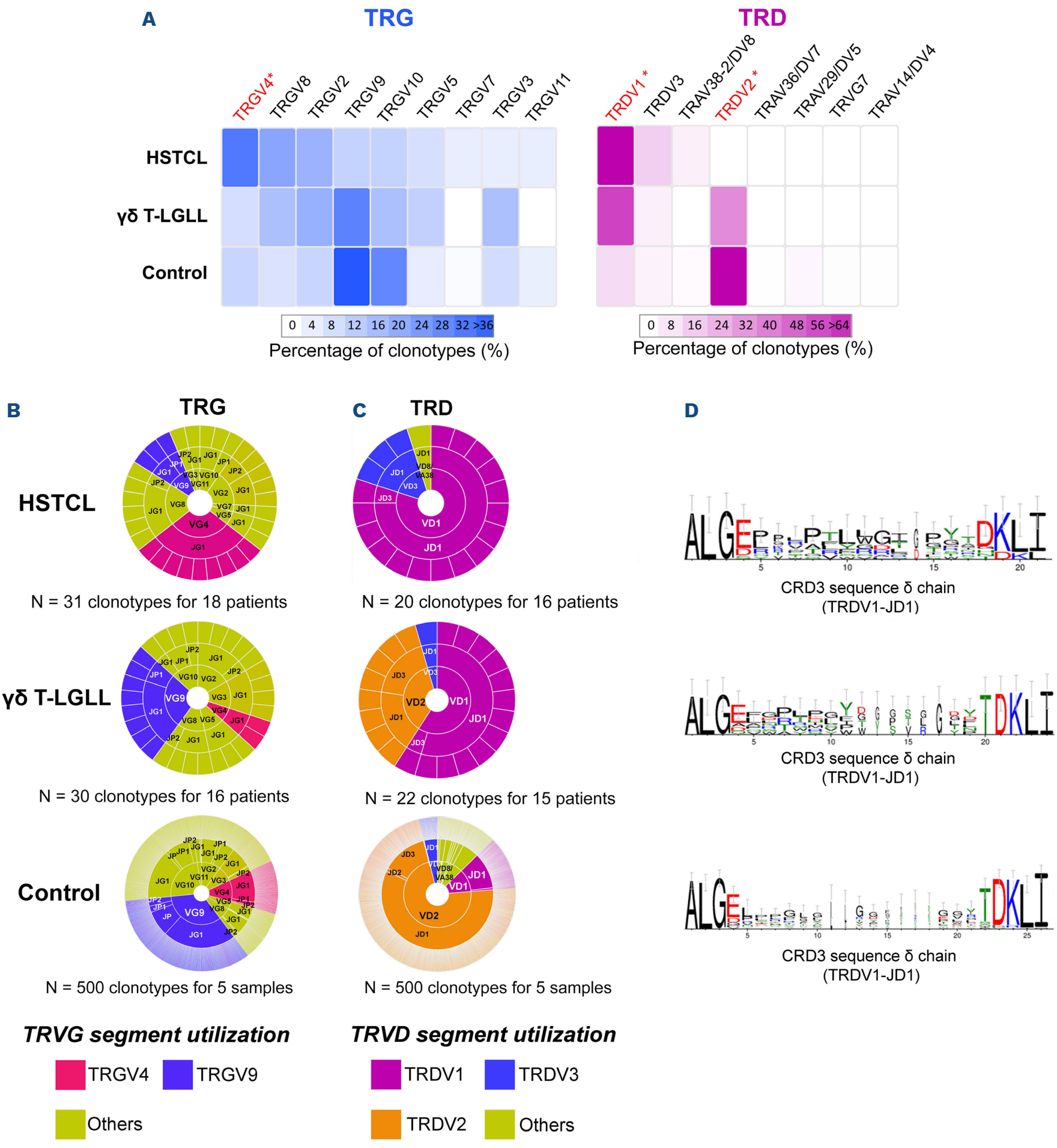

Figure 2. Comparison of *TRG* and *TRD* rearrangement profiles in dominant clonotypes from hepatosplenic T-cell lymphoma, γδT-cell large granular lymphocytic leukemia and a polyclonal control group. (A) Heatmap showing frequency of segment usage for *TRG* and *TRD* loci in the hepatosplenic T-cell lymphoma (HSTCL) group, compared to the γδT-large granular lymphocytic leukemia (LGLL) group and the polyclonal control group. **TRGV4* is significantly overrepresented in HSTCL clonotypes *versus* LGLL and *versus* control clonotypes; *TRDV1* is overrepresented in HSTCL and LGLL clonotypes *versus* control clonotypes; *TRDV2* is overrepresented in LGLL and control clonotypes *versus* HSTCL clonotypes ($P<0.05$). (B) Sunburst representation of the utilization of *TRG* segments in the dominant clonotypes identified in the HSTCL, LGLL and control groups. (C) Sunburst representation of the utilization of *TRD* segments in the dominant clonotypes identified in the HSTCL, LGLL and control groups. (D) Comparison of the CDR3 amino-acid sequences of VD1-JD1 clonotypes in the HSTCL, LGLL and control groups. TRG: T-cell receptor gamma; TRD: T-cell receptor delta.

biased *TRG* and *TRD* gene usages that distinguish them from LGLL (Figure 2).

TRG and *TRD* gene rearrangements were sequenced according to the two-step polymerase chain reaction EuroClonality next-generation sequencing protocols on a MiSeq platform (Illumina) and analyzed with the Vidjil tool.[13] We compared the dominant clonotypes from 20 HSTCL and 16 γδT-LGLL cases with an *in silico* polyclonal control composed of the top 100 clonotypes from five healthy donors (Figure 2A). Regarding the *TRG* locus (Figure 2B), 31 clonotypes were detected in 18 HSTCL cases (90%) with a predominance of rearrangements involving the *TRGV4* gene in nine cases (50%). The *TRGV4* gene was significantly more prevalent in HSTCL clonotypes (29%) than in γδT-LGLL (7%; *P*=0.043) and the control group (9%; *P*=0.002). In γδT-LGLL and the control group, the most represented gene was *TRGV9*, occurring in eight LGLL clonotypes (27%) and in 37% of control clonotypes, which is significantly higher than in the HSTCL group (10%) (*P*=0.002). Regarding the *TRD* locus (Figure 2C), we identified 20 clonotypes in 16 HSTCL samples and 22 clonotypes in 15 γδT-LGLL samples. We observed a higher number of clonotypes using the *TRDV1* gene in HSTCL and γδT-LGLL cases than in the control group (*P*<0.001 for both). Additionally, 13 HSTCL cases (81% of γδT-positive cases) showed a VD1-JD1 rearrangement, while this occurred in eight γδT-LGLL cases (50%). To a lesser extent, HSCTL clonotypes used the *TRDV3* gene more frequently than γδT-LGLL did (*P*=0.050). In contrast, *TRDV2* was more frequently represented in the control group (80% of clonotypes) and in γδT-LGLL patients (47%) than in the HSTCL group (0%) (*P*<0.001 and *P*=0.004, respectively). Finally, only two HSTCL patients had *TRB* rearrangements, corresponding to the two samples expressing αβTCR identified by flow cytometry. Overall, we demonstrate a higher prevalence of *TRGV4* and *TRDV1* segment utilization in HSTCL patients. This observation aligns with previous phenotyping investigations that showed a predominant expression of Vδ1 chain in HSTCL and in normal splenic γδ T cells.[14,15] Moreover, tumor infiltrating Vδ1 T cells often exhibit immunosuppressive effects, in contrast to classical Vγ9Vδ2 T cells, which mainly exist in peripheral blood and have strong anti-tumor effects in various types of tumors.[15] In line with the findings of Teramo *et al.*, LGLL exhibited a higher frequency of *TRGV9* and *TRDV2* genes in their *TRG* and *TRD* rearrangements, respectively. Consistently, the preferential expression of the Vγ9Vδ2 phenotype in γδT-LGLL appears to mimic the spectrum of normal T cells in the peripheral blood of healthy subjects and is associated with less symptomatic presentation in LGLL.[12] In HSTCL clonotypes, we observed similar peptide amino-acid sequences within the CDR3 region of the δ chain, particularly involving VD1-JD1 rearrangements (Figure 2D and *Online Supplementary Table S2*). These CDR3 sequences were shorter and showed less diversity than those observed in VD1-JD1 clonotypes from subjects with γδT-LGLL and healthy donors, suggesting a role for antigenic recognition as an early event in HSTCL lymphomagenesis.

In conclusion, HSTCL may originate from a clonally selected γδT population with stereotyped TCR, through the acquisition of somatic mutations leading to JAK/STAT pathway deregulation and chromatin modifications. We provide a more comprehensive characterization of HSTCL patients compared to those with γδT-LGLL, highlighting a specific oyster-shell morphology and restricted *TRG* and *TRD* segment usages in HSTCL. Together, these findings may serve as a valuable tool for distinguishing HSTCL from other γδT proliferations and could potentially reduce the need for more invasive procedures such as splenectomy or liver biopsy.

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Disclosures

No conflicts of interest to disclose.

Contributions

AD, SB, and CP designed the research and wrote the paper. FT performed T-cell receptor sequencing bioinformatics analysis. MP described the cytological pattern. AD, SB, JG, and MP reviewed cytological data. FL-G reviewed histological data. FG, LC, LB, PL, CLL, PC-L, A-CG, CB, ER, ED, CF, FB-J, SW, and VB performed the initial investigations and addressed the cases for constitution of the cohort. TL managed the patients with large granular lymphocytic leukemia. MR provided flow cytometric data. AD, SB, TF, and CP analyzed the molecular data. All the authors critically reviewed the paper.

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Data-sharing statement

Original data including metadata, images and fastq files are available upon request from cedric.pastoret@chu-rennes.fr.

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