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Case report

Emergence of a STAT3 mutated NK clone in LGL leukemia

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ABSTRACT

Large granular lymphocyte (LGL) leukemia is a chronic clonal lymphoproliferative disorder. Here, a T-LGL leukemia patient developed NK-LGL leukemia with residual leukemic T-LGL. TCRV β usage and CDR3 sequence drifts were observed with disease progression. A STAT3 S614R mutation was identified in NK but not T-cells in the mixed leukemic stage. Multiple, non-dominant T-cell clones with distinct STAT3 mutations were present throughout. Our results suggest that T and NK-LGL leukemia may share common pathogenesis mechanisms and that STAT3 mutation alone is insufficient to bring about clonal expansion. Mutational and immunological monitoring may provide diagnostic and therapeutic significance in LGL leukemia.

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1. Introduction

Common clinical manifestations of large granular lymphocyte leukemia (LGL leukemia) include cytopenias, recurrent infections, splenomegaly, and autoimmune disorders [1,2]. Diagnosis can be difficult, and is usually based on clinical presentation, persistent peripheral LGL lymphocytosis, and morphologic/immunophenotypic analysis [2]. For T-LGL leukemia, a T-cell receptor (TCR) gene rearrangement study is performed to confirm the presence of the monoclonal T-cell population. Determining the clonality of NK-LGL cells is challenging. An abnormal killer-cell Ig-like inhibitory receptor (KIR) expression pattern has been reported in some of the NK-LGL leukemia patients, although this is not routinely employed [3].

Chronic exposure to antigen or virus has been postulated to be the initial trigger in LGL leukemia pathogenesis [4,5]. Recent studies demonstrated that JAK/STAT pathway deregulation may promote clonal expansion of the activated LGL population [6,7]. To further understand LGL leukemia pathogenesis, we identified a patient with a distinct clinical course. The CDR3 sequences, TCRV β profiles, STAT3 mutation profile and the underlying signaling pathway were examined throughout the disease.

2. Materials and methods

The Institutional Review Board of Hershey Medical Center, Pennsylvania State University approved this study. The patient's peripheral blood mononuclear cells were collected between 2002 and 2013. For TCR deep sequencing, the TCR CDR3 β regions were amplified and sequenced by Adaptive Biotechnologies Corp (Seattle, WA) using the ImmunoSEQ assay on cDNAs [8]. Data was analyzed using the ImmunoSEQ analyzer toolset [9]. Real-time quantitative PCR (qRT-PCR) was employed to examine the TCRV β profile, using primers and methods described previously [10], except that SybrGreen (Life Technologies) was used and target specificity was confirmed within the IMGT primer database [11]. Deep sequencing results for one clone were validated using PCR-based CDR3 sequencing with primers designed from the IMGT primer database (TBV9, 5'-CAC TCT GAA CTA AAC CTG A-3'; CB1A, 5'-GGG TGT GGG AGA TCC TG(C)-3'). Conventional Sanger STAT3 mutation testing was carried out as previously described by Koskela et al. [7]. Primer pairs were designed to cover exons 20–21 encoding the STAT3 Src Homology 2 (SH2) domain [6,7]. Droplet digital PCR (ddPCR) assays were designed to detect individual mutations D661Y, Y640F and S614R. Assays were designed to the following context sequences: D661Y GGATATTGG-TAGCAT(A/C)CATGATCTTATAGCC, Y640F AGCTGCTGCTTTGTG(A/T)ATGGTTCCACGGACT, and S614R GACGCCTCCTTCTTT(T/G)CTGC TTTCCTGAAT. Assays were performed in duplicate on the QX200 droplet reader and generator (Bio-Rad, Hercules, CA). Western immunoblotting utilized antibodies to STAT3 (#9139), pY705-STAT3 (#9131), MCL-1 (#5453) and GAPDH (#2118) (Cell

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Signaling, Danvers MA). Intensities of the protein bands were determined using ImageJ software [12].

3. Results and discussion

3.1. Case summary

A 77-year-old male presented in 2002 with mild macrocytic anemia and splenomegaly. Based on the peripheral blood morphology, bone marrow biopsy, flow cytometry and positive TCR gene rearrangement assay, CD3+/CD8+ T-LGL leukemia was diagnosed. The patient remained asymptomatic for the next nine years. He did not require any therapy other than routine follow-up, which showed stable blood counts (Table 1A). In 2011, his follow-up CBC revealed worsening anemia and moderate neutropenia with increased lymphocytosis. Repeated flow cytometry showed a marked increase in the CD3-/CD16+/CD56- population that represents the NK-cell phenotype, with a smaller CD3+/CD8+ T-cell population. These results suggested that his leukemia had evolved to NK-predominant disease with a residual of previous T-LGL leukemia (Table 1A). Despite the progression of his leukemia, he has remained asymptomatic and clinically stable to date, and no treatment has been required. He has been monitored every three months with CBCs showing stable cytopenias.

3.2. TCRVβ profiling and clonal CDR3 sequences throughout disease evolution

Antigen exposure has been postulated as the initial stimulus for CTL expansion in T-LGL leukemia pathogenesis [4]. We employed qRT-PCR to study the dynamic change of TCRVβ profile using primers designed to amplify each of the human TCRVβ genes (HBV) [10]. As shown in Table 1A, shortly after his initial diagnosis there was a predominant expression in the TCRVβ9 region with smaller expansions in the TCRVβ3 and TCRVβ21 regions. TCRVβ3 and TCRVβ21 usages persisted and later became predominant, while TCRVβ9 expression was lost, revealing a drift in TCRVβ usage that correlated with the T-LGL to NK-LGL phenotypic switch.

CDR3 is a hypervariable region on the TCR that is critical for antigen recognition and clonal T-cell expansion. Sequencing of this region in T-LGL leukemia was recently enabled by utilizing deep sequencing technology [13]. Three CDR3 sequences were detected at very high frequencies in the diagnostic T-LGL leukemia sample of 2003 (Table 1B). All three clones persisted throughout the disease; however, the clone represented by the CDR3 sequence CASSTGDNQPQHF, which had the highest copy number initially, was significantly decreased to an extremely low copy number when the NK-LGL clone emerged. Meanwhile, the other two clones became more dominant as the disease evolved, similar to oligoclonal TCRVβ distribution depicted in Table 1A. The CDR3

sequences identified in this case appeared to be unique compared to the sequences reported in other T-LGL leukemia studies [13,14].

Clonal drift was previously reported in one third of T-LGL leukemia cases, although it is not strongly associated with clinical features [15]. The worsening of cytopenias, such as this patient's anemia at later dates, implies an important role of clonal drift in disease evolution. Such drift may precede changes in the disease course. Therefore, long-term immunophenotypic monitoring may provide clinical relevance and guide medical management.

3.3. STAT3 mutation in NK-LGL leukemia during disease progression

It has been reported that 40–70% of T-LGL and 30% of NK-LGL leukemia patients carry activating STAT3 mutations [6,7]. To investigate whether a deregulated STAT3 pathway was associated with the patient's disease progression, we searched for mutations in the STAT3 SH2 domain before and after the T-LGL to NK-LGL phenotype switch. A STAT3 S614R mutation was identified by Sanger sequencing later in the illness in the NK-cells, but not the T-cells (Fig. 1A, data not shown for early stage). An elevated phosphorylated STAT3 level and an increase in its transcriptional target MCL-1 were observed in the mutant samples (Fig. 1B), consistent with enhanced transcriptional activity of the mutant protein.

In order to determine if the S614R-mutated clone had been present all along, we tested unsorted genomic DNA samples from 2003 and 2011 for S614R and for two common mutations Y640F and D661Y by ddPCR. Both samples contained approximately 35,000 copies of the STAT3 locus, yielding approximately 9000 positive droplets for the wild-type allele. All reported positive droplet and percentage counts for mutations (Fig. 1C) exceeded the 1–2 positive droplets found in similar amounts of normal DNA by at least 10-fold (not shown). S614R mutations were not detected in the earlier sample (no copies mutated in a background of roughly 35,000 wild-type copies). Small but non-negligible amounts of D661Y and Y640F mutations suggested the presence of 8.52% and 0.68% clones, respectively, assuming heterozygous mutation. These contracted to roughly 0.1% (1:500 cells) in the follow-up sample, shadowing the decrease in the T cell

Table 1B

The most frequent CDR3 sequences detected by deep sequencing and number of unique sequences/total productive reads (%) in different disease stages.

CDR3 sequences	Immunodominant clonotypes		
	Vβ3: CASSQWYQYF (%)	Vβ9: CASSTGDNQPQHF (%)	Vβ21: CARSSDRASYEQYF (%)
2003	16.2	17.5	7.8
2012	33.0	0.5	15.8

The sequence of CASSTGDNQPQHF was validated by PCR-based sequencing.

Table 1A

Representative CBC and flow cytometry results, and TCRVβ clonality profile throughout the disease course.

	CBC			Flow cytometry								TCRVβ Usage		
	WBC	ANC	Lymph	CD3		CD8		CD16		CD3-/CD16+		Vβ3	Vβ9	Vβ21
	10 ³ /mcl	10 ³ /mcl	10 ³ /mcl	(%)	#	(%)	#	(%)	#	(%)	#	(%)	(%)	(%)
2003	8.4	2.9	4.5	88	3.9	68	3.0	23	1	ND	ND	8.8	48.7	4.9
2004	7.1	2.1	3.5	ND	ND	ND	ND	ND	ND	ND	ND	10.7	27.7	7.2
2011	9.0	0.9	6.9	33	3.0	23	2.1	68	6.1	67	6.0	29.4	4.0	14.8
2012	7.0	0.9	6.1	27	2.1	18	1.4	74	5.8	74	5.8	20.9	2.9	18.5

For TCRVβ profile, qRT-PCR was performed on cDNA synthesized from RNA extracted at different time points (as indicated) during the disease course. Percentage (%) reflecting single family expression by the sum of all measured Vβ-families is shown here. ND: Not done.

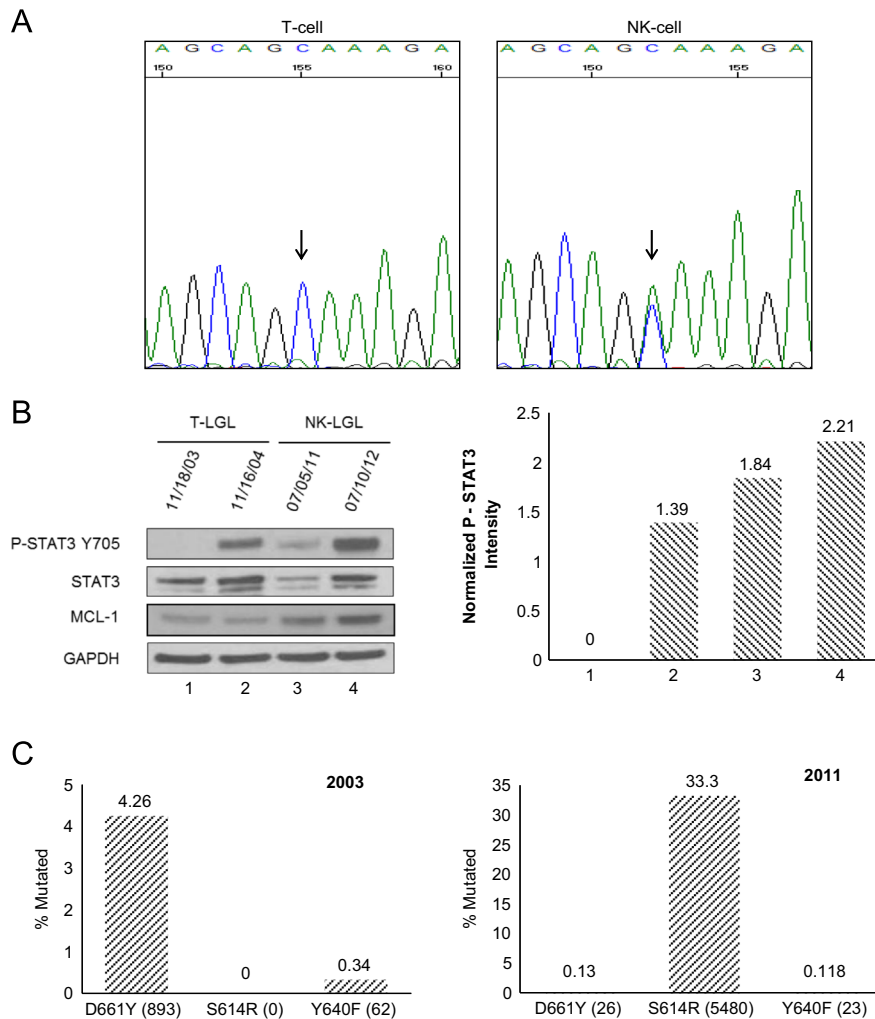


Fig. 1. STAT3 S614R mutation identified in NK-cells during later disease course. (A) PBMCs from a single sample (collected 7/10/2012) were subjected to negative selection in order to isolate purified T-cell and NK-cell populations. RNAs extracted from T-cell (left panel) and NK-cell (right panel), respectively, were used for RT-PCR to obtain cDNAs for STAT3 sequencing. Sequencing chromatograms are shown here. Arrows indicate a C→A point mutation (S614R) that is only seen in the NK cell population. (B) Left panel: Western blot analysis performed on cell lysates prepared from total PBMCs obtained throughout the disease course. Labels indicate dominant phenotype at the time of sample collection on the indicated dates. Right panel: Histogram of normalized P-STAT3 Y705 intensity. Band intensity was measured using ImageJ and P-STAT3 Y705 signal was normalized against total STAT3 level. Phosphorylation of STAT3 Y705 and levels of the STAT3 target MCL-1 increased as the disease progressed. (C) Mutation percentage of PBMC DNA from sample collected in 2003 (left) versus 2011 (right) as determined by ddPCR. Bracketed numbers are the number of positive droplets for that mutation in the assay.

compartment. In contrast, S614R dominated in the follow-up specimen with a mutation rate of 33.3% and a clone size of roughly 67%, which is identical to the CD3⁻/CD16⁺ population reported in Table 1A. Therefore, this patient developed a dominant NK-LGL clone harboring the S614R mutation, while a population of cells with a strongly-activating Y640F mutation remained a minority clone. This clearly suggests that the capacity of particular STAT3 mutations to drive clonal dominance cannot be predicted *a priori*, rather it is likely to be dependent upon the genetic context of these mutations such as co-existent activating mutations or loss of suppressive checkpoint mechanisms.

STAT3 S614R was previously described exclusively in T-LGL leukemia [6]. Our findings here not only complement the known mutational mechanism for NK-LGL leukemogenesis, but also suggest that T and NK-LGL leukemia can result from the same underlying pathological process, with mutations in STAT3 able to support the expansion of either cell type.

In 2009, the World Health Organization recognized a provisional category of chronic lymphoproliferative disorder of NK cells to distinguish it from T-cell LGL leukemia as well as aggressive NK-LGL leukemia [16]. The clinical and molecular data presented

here suggests that this distinction amongst the chronic LGL diseases may need to be reconsidered.

Authorship contributions

Yiyi Yan designed and performed experiments, analyzed the data, wrote the manuscript and provided figures. Thomas Olson designed the TCR qRT-PCR experiments and STAT3 mutation detection, analyzed the data, and wrote the manuscript. Susan Bell Nyland designed the TCR deep sequencing experiments, analyzed the data, wrote the manuscript and provided figures. David Feith designed the experiments, analyzed the data, and wrote the manuscript. Thomas P. Loughran Jr. designed the experiments, analyzed the data and wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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References

- [1] Loughran Jr. TP, Kadin ME, Starkebaum G, Abkowitz JL, Clark EA, Distche C, et al. Leukemia of large granular lymphocytes: association with clonal chromosomal abnormalities and autoimmune neutropenia, thrombocytopenia, and hemolytic anemia. *Ann Intern Med* 1985;102:169–75.
- [2] Sokol L, Loughran Jr. TP. Large granular lymphocyte leukemia. *Curr Hematol Malig Rep* 2007;2:278–82.
- [3] Zambello R, Semenzato G. Natural killer receptors in patients with lymphoproliferative diseases of granular lymphocytes. *Semin Hematol* 2003;40:201–12.
- [4] Yang J, Epling-Burnette PK, Painter JS, Zou J, Bai F, Wei S, et al. Antigen activation and impaired Fas-induced death-inducing signaling complex formation in T-large-granular lymphocyte leukemia. *Blood* 2008;111:1610–6.
- [5] Loughran Jr. TP, Hadlock KG, Yang Q, Perzova R, Zambello R, Semenzato G, et al. Seroreactivity to an envelope protein of human T-cell leukemia/lymphoma virus in patients with CD3- (natural killer) lymphoproliferative disease of granular lymphocytes. *Blood* 1997;90:1977–81.
- [6] Jerez A, Clemente MJ, Makishima H, Koskela H, Leblanc F, Peng Ng K, et al. STAT3 mutations unify the pathogenesis of chronic lymphoproliferative disorders of NK cells and T-cell large granular lymphocyte leukemia. *Blood* 2012;120:3048–57.
- [7] Koskela HL, Eldfors S, Ellonen P, van Adrichem AJ, Kuusanmaki H, Andersson EI, et al. Somatic STAT3 mutations in large granular lymphocytic leukemia. *N Engl J Med* 2012;366:1905–13.
- [8] Robins HS, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O, et al. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood* 2009;114:4099–107.
- [9] Robins H, Desmarais C, Matthis J, Livingston R, Andriesen J, Reijonen H, et al. Ultra-sensitive detection of rare T cell clones. *J Immunol Methods* 2012;375:14–9.
- [10] Ochsenreither S, Fusi A, Busse A, Nagorsen D, Schrama D, Becker J, et al. Relative quantification of TCR Vbeta-chain families by real time PCR for identification of clonal T-cell populations. *J Transl Med* 2008;6:34.
- [11] Lefranc MP, Giudicelli V, Ginestoux C, Bodmer J, Muller W, Bontrop R, et al. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res* 1999;27:209–12.
- [12] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;9:671–5.
- [13] Clemente MJ, Przychodzen B, Jerez A, Dienes BE, Afable MG, Husseinzadeh H, et al. Deep sequencing of the T-cell receptor repertoire in CD8+ T-large granular lymphocyte leukemia identifies signature landscapes. *Blood* 2013;122:4077–85.
- [14] Wlodarski MW, O'Keefe C, Howe EC, Risitano AM, Rodriguez A, Warshawsky I, et al. Pathologic clonal cytotoxic T-cell responses: nonrandom nature of the T-cell-receptor restriction in large granular lymphocyte leukemia. *Blood* 2005;106:2769–80.
- [15] Clemente MJ, Wlodarski MW, Makishima H, Viny AD, Bretschneider I, Shaik M, et al. Clonal drift demonstrates unexpected dynamics of the T-cell repertoire in T-large granular lymphocyte leukemia. *Blood* 2011;118:4384–93.
- [16] Lim MS, de Leval L, Quintanilla-Martinez L. Commentary on the 2008 WHO classification of mature T- and NK-cell neoplasms. *J Hematopathol* 2009;2:65–73.