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Original Article

Classification of NK-large granular lymphocytic leukemia by CD56 expression

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Abstract

NK-large granular lymphocytic leukemia (NK-LGLL) is a rare chronic lymphoproliferative disorder and displays heterogeneity that remains insufficiently defined. CD56 plays a pivotal role in NK-cell maturation linked to cytotoxicity. However, whether CD56 might be associated with distinctive characteristics in NK-LGLL has not been determined. Hence, this study aims to explore potential associations between CD56 and clinical and biological features in 47 patients with NK-LGLL. Above all, anemia (57.4%) was the most prevalent symptom. Patients treated with immunosuppressive therapy showed a favorable outcome with 87.0% achieving remission. Furthermore, when stratifying patients by CD56 expression on tumor cells, the subset of 28 patients (59.6%) with diminished CD56 expression was frequently relevant to symptomatic disease (92.9% vs 15.8%, $P < .001$), comprising anemia (85.7% vs 15.8%, $P < .001$), neutropenia (67.9% vs 0.0%, $P < .001$), and splenomegaly (42.9% vs 10.5%, $P = .024$). Additionally, this subset demonstrated exclusive STAT3 mutation (61.9% vs 0.0%, $P = .003$), elevated CD161 levels (54.5% vs 0.0%, $P < .001$), and bone marrow fibrosis (92.3% vs 50.0%, $P = 0.006$). Furthermore, they showed shorter time to first treatment (TTFT) (4-year TTFT: 66.7% vs 100.0%, $P = .083$) and first-line progression-free survival (PFS) (median PFS: 26.3 months vs not reached, $P = .112$). Overall, our data indicate that NK-LGLL patients with diminished CD56 expression represent a more aggressive subset compared to those with normal CD56 levels, underscoring the significance of CD56 as a potential prognostic marker and advancing our understanding of the underlying pathogenesis of NK-LGLL.

Key words: leukemia; large granular lymphocytic; killer cells; natural; biomarkers; immunology; genetics; therapy.

Implications for practice

Our findings indicate an indolent course and excellent response to immunosuppressive therapies of NK-large granular lymphocytic leukemia (NK-LGLL), significantly advancing the classification and unveiling the distinct prognostic of patients with NK-LGL, which will pave the way for more refined treatment strategies and a deeper exploration of the underlying mechanisms driving this complex disease in the future.

Introduction

Large granular lymphocyte leukemia (LGLL) is a rare and heterogeneous disorder characterized by clonal expansion of cytotoxic lymphocytes. Generally, LGLL can be categorized according to the immunophenotype of tumor cells as T-large granular lymphocytic leukemia (T-LGLL) and NK-large granular lymphocytic leukemia (NK-LGLL), with NK-LGLL constituting roughly 10% of the total disease.[1] Despite numerous researches have demonstrated the sharing of clinical features and pathogenesis between the 2 subtypes,[2-6] LGLL patients display extremely different clinical manifestations, treatment responses, and quite diverse biological features. Furthermore, recent studies suggest that the expression of CD8 or γδTCR, loss of TCRVδ2 on T-LGLs, and *STAT3* mutation may indicate a more symptomatic disease and poorer survival in patients with T-LGLL, underscoring the heterogeneity of the disease.[2,6-8] In contrast, the heterogeneity of clinical and

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biological characteristics in NK-LGLL remains unclear as its rarity.

NK-large granular lymphocytes (LGLs) are typically considered to be mature and activated NK cells (CD2+/CD3–/CD16+ or CD56+) demonstrating more potent cytotoxic immunophenotypes than the health.[9,10] Physiologically, the expression of CD56 indicated the transformation from immature NK cells to mature NK cells.[11] To further, mature NK cells could be divided into 2 subsets, CD56dim and CD56bright. The CD56dim NK-cell subset exhibits elevated levels of Ig-like NK receptors (KIRs) and CD16 conferring enhanced cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) compared to CD56bright NK cells. In contrast, CD56bright NK cells prefer to produce higher levels of cytokines, including TNF-β, IFN-γ, and IL-10, in response to monocyte stimulation and for interacting with other immune cells.[12,13] Despite these distinctions in physically, the clinical and biological difference between CD56 dim or bright expression NK-LGLL has yet to be fully determined. Toward that end, in this study, we conducted an analysis of a large cohort with 47 NK-LGLL patients, finding that patients presenting CD56 dim or negative expression on NK-LGLs had more severe symptoms and worse outcomes.

Methods

Study patients

Between January 2014 and July 2023, 47 patients diagnosed with NK-LGLL were collected from the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences, and Peking Union Medical College. All the patients met the recommended diagnostic criteria for LGLL,[14] which was based on persistent LGLs in peripheral blood showing CD3–/CD16+, or CD56+ immunophenotypic pattern. In patients with a circulating NK clone <0.5 × 10^9/L or without KIRs testing, the disease was confirmed by a comprehensive consideration of clinical or biological features, and bone marrow biopsy to exclude other hematological malignancies. All cases involved in this study were approved by the ethics committees of the Chinese Academy of Medical Sciences & Blood Disease Hospital, and patients' informed consent was obtained in accordance with the Declaration of Helsinki.

Flow cytometry analysis

Flow cytometric immune phenotypic analyses were done on bone marrow or peripheral blood samples by multiparameter flow cytometry (Navios, Beckman Coulter), and analyses were performed using the Kaluza analysis 2.0. NK LGLL was defined by flow cytometry analysis using common surface markers showing CD3–/CD16+, or CD56+ pattern. Other surface markers of NK LGL were also evaluated in a part of patients, including CD2, CD3, CD5, CD7, CD8, CD45RA, CD45RO, CD57, CD94, CD161, Perforin, and granzyme B (GramB). Cells were considered antigen partial positive if antibody staining was between 20% and 80%, and considered positive or negative if >80% or <20% had positive staining. Analysis of KIR expression was carried out using the following markers: CD158a, CD158b, CD158e, CD158f, and CD158i. Either restrictive or lack of expression was able to indicate clonality.[15]

Next generation sequencing

Next generation sequencing was performed using 125 lymphoma-associated genes (Supplementary Table S1). Gene aberrations that were relevant to LGLL posted in present studies were almost covered in both panels, including *STAT3*, *STAT5B*, *TET2*, *KMT2D*, *DNMT3A*, *JAK3*, and *TNFAIP*.[16,17] Genomic DNA was extracted from bone marrow aspirate. Sequencing libraries were developed using Agilent SureSelect Human All Exon kit (Agilent Technologies), and the target-enriched libraries were sequenced on Illumina HiSeq 2000 sequencer (Illumina). An average sequencing depth of 2000× was achieved.

Treatments and evaluation of outcomes

Following were the indications of therapy: ANC <0.5 × 10^9/L or neutropenia accompanied by recurrent infections, HB <100g/L, PLT <50 × 10^9/L, symptomatic autoimmune disorders, and any severe B symptoms. Efficacy assessment criteria were according to the Eastern Cooperative Oncology Group 5998 T-LGLL trial[18] after at least 3 months of therapy: complete response (CR) was defined as absolute neutrophil count (ANC) >1.5 × 10^9/L, hemoglobin (HB) > 110 g/L, platelet count (PLT) >100×10^9/L; Partial response (PR) was defined as an improvement in blood counts ANC >0.5 × 10^9/L; HB increased by >10 g/L; PLT >50 × 109/L, and the absence of required transfusions. Progressive disease (PD) was defined as a worsening of hematologic parameters (a decrease in 20 g/L of HB and less than 100 g/L, or decrease in 0.5 × 10^9/L of ANC and less than 1.0 × 10^9/L, or decrease in 20 × 10^9/L of PLT and less than 100 × 10^9/L, or required transfusions) or findings of organomegaly such as hepatosplenomegaly are detected in patients previously achieving PR or CR. We used abdominal ultrasound to assess spleen size, with splenomegaly being defined as a longitudinal diameter greater than 13 centimeters.

Statistical analysis

Categorial variables compared between patient groups were performed by Fisher's exact test or chi-square test. Comparisons of quantitative variables were Mann-Whitney *U* test. Overall survival (OS) was calculated from the date of diagnosis to death irrespective of causes or the last follow up for censored patients. Progression-free survival (PFS) was identified as the duration of the date initiating treatment and the date of disease progression or death, and the last visit for censored one. Time to first treatment (TTFT) referred to the duration between the interval between the initial diagnosis and the commencement of the first treatment intervention. Survival curves were estimated using the Kaplan-Meier method and compared using the log-rank test. A 2-side *P* value < .05 was considered significant. The above statistical analyses were conducted on IBM SPSS Statistics 26.

Results

Clinical and biological features of NK-LGLL patients

The clinical and biological features of the 47 NK-LGLL cases arevsummarized in Table 1. The median age at diagnosis was 52 (range 23-82). Sex ratio M/F was 1.9 (31/16). More than half of the patients (61.7%) presented symptoms (including cytopenia and splenomegaly) at the time of

Table 1. Clinical and biological features of 47 NK-LGLL patients and the diminished/ normal CD56 groups

Characteristic	NK-LGLL (*N* = 47)	Diminished CD56 group (*N* = 28)	Normal CD56 group (*N* = 19)	*P* value (diminished CD56 group vs normal CD56 group)
Median follow-up [min; max]-month	56.2 [6.7;112.3]	45.8 [6.7; 112.3]	56.4 [12.8; 107.2]	.678
Age at diagnosis-years [min; max]	52 [23; 82]	53 [25; 82]	52 [23; 70]	.522
Gender Male	31/47 (66.0%)	25/28 (89.3%)	6/19 (31.6%)	<.001
Median LGL [min; max]- × 10^9/L	3.7 [0.04;16.9]	0.9 [0.04; 10.4]	7.6 [0.2; 16.9]	<.001
Need for treatment	27/47 (57.4%)	24/28 (85.7%)	3/19 (15.8%)	<.001
Symptoms at first visit	29/47 (61.7%)	26/28 (92.9%)	3/9 (15.8%)	<.001
HB <120 g/L	27/47 (57.4%)	24/28 (85.7%)	3/19 (15.8%)	<.001
HB <90 g/L	18/47 (38.3%)	18/28 (64.3%)	0/19 (0.0%)	<.001
ANC <1.5 × 10^9/L	19/47 (40.4%)	19/28 (67.9%)	0/19 (0.0%)	<.001
ANC <0.5 × 10^9/L	2/47 (4.3%)	2/28 (7.1%)	0/19 (0.0%)	.350
PLTs <100 × 10^9/L	6/47 (12.8%)	5/28 (17.9%)	1/19 (5.3%)	.410
Splenomegaly	14/47 (29.8%)	12/28 (42.9%)	2/19 (10.5%)	.024
ECOG-PS				.105
0	34/47 (72.3%)	17/28 (60.7%)	17/19 (89.5%)	
1	10/47 (21.7%)	9/28 (32.1%)	1/19 (5.3%)	
2	3/47 (6.5%)	2/28 (7.1%)	1/19 (5.3%)	
Autoimmune diseases	7/47 (14.9%)	6/28 (21.4%)	1/19 (5.3%)	.267
PRCA	6/43 (14.0%)	6/26 (23.1%)	0/17 (0.0%)	.092
Secondary primary malignancies	2/47 (4.3%)	0/28 (0.0%)	2/19 (10.5%)	.158
LDH increased	13/42 (31.0%)	11/24 (45.8%)	2/18 (11.1%)	.016
CD2 expression	41/41 (100.0%)			
CD3 expression	0/47 (0.0%)			
CD8 expression	11/45 (24.5%)	4/24 (14.3%)	7/17 (41.2%)	.093
CD16 expression	37/47 (78.7%)	24/28 (85.7%)	13/19 (68.4%)	.290
CD56 expression				
Positive	22/47 (46.8%)			
Partial positive	8/47 (17.0%)			
Negative	17/47 (36.2%)			
CD56 MIF				
Bright or moderate	19/22 (86.4%)			
Dim	3/22 (13.6%)			
CD57 expression	27/43 (62.8%)	14/27 (51.9%)	13/16 (81.3%)	.101
CD94 expression	30/38 (78.9%)	14/21 (66.7%)	16/17 (94.1%)	.096
CD161 expression	12/38 (31.6%)	12/22 (54.5%)	0/16 (0.0%)	<.001
Perforin	35/41 (85.4%)	22/25 (88.0%)	13/16 (81.3%)	.886
Granzyme B	33/41 (80.5%)	21/25 (84.0%)	12/16 (75.0%)	.760
KIR expression				.832
CD158a/b/e/f/i negative	23/37 (62.2%)	14/22 (63.6%)	9/15 (60.0%)	
Restrictive expression	14/37 (37.8%)	8/22 (36.4%)	6/15 (40.0%)	
CD158i	10/37 (27.0%)	7/22 (31.8%)	3/15 (20.0%)	
CD158a	3/37 (8.1%)	2/22 (9.1%)	1/15 (6.7%)	
CD158b	2/37 (5.4%)	0/22 (0.0%)	2/15(13.3%)	
STAT3 mutation	13/32 (40.6%)	13/21 (61.9%)	0/11 (0.0%)	.003
STAT5B mutation	0/32 (0.0%)			
LGL in bone marrow [min; max] %	21.1 [2.4; 51.1]	17.3 [2.4; 47.2]	22.8 [6.2; 51.1]	.135
Bone marrow fibrosis				.006
0	10/42 (23.8%)	2/26 (7.7%)	8/16 (50.0%)	
1	30/42 (71.4%)	22/26 (84.6%)	8/16 (50.0%)	
2	2/42 (4.8%)	2/26 (7.7%)	0/16 (0.0%)	

Abbreviations: ANC, absolute neutrophil count; ECOG-PS, Eastern Cooperative Oncology Group performance status; HB, hemoglobin;KIR, Ig-like NK receptors; LGL, large granular lymphocyte; PLT, platelet; PRCA, pure red blood cell aplastic anemia.

visit, among which anemia (HB < 120g/L) was the primary clinical manifestations observed in 57.4%, when moderate to severe anemia accounted for 38.3% of patients. Besides, neutropenia (ANC < 1.5 × 10^9/L) was founded in 40.4% of the patients, and severe neutropenia (ANC <0.5 × 10^9/L) happened in merely 4.3%. Thrombocytopenia was relatively rare in LGLL, occurring in 12.8% of patients. The median NK-LGLs counts was 3.7 × 10^9/L (range 0.04-16.9 × 10^9/L). Concurrent autoimmune disorders were present in 14.9% of patients, with 4 cases being rheumatoid arthritis. Pure red cell aplasia (PRCA) was found in 14.0%. Bone marrow examination by flow cytometry revealed that NK-LGLs accounted for 21.1% (range 2.4%-51.1%) of nuclear cells and 71.4% of the patients had grade 1 bone marrow fibrosis.

Regarding immunophenotypes, all patients demonstrated an expansion of CD3–/CD16+ and/or CD56+ NK cells. The expressions of mature NK cell markers were heterogenous, including CD8 (24.5%), CD16 (78.7%), CD56 (46.8%), CD57 (62.8%), CD94 (78.9%), CD161 (31.6%), Perforin (85.4%), and GramB (80.5%). To identify clonality of tumoral NK-LGLs, we examined the KIR repertoire in 37 patients. Among them, 23 of 37 (62.2%) did not exhibit any of the 5 assessed KIR, when the remaining (37.8%) showed a restrictive pattern, predominantly of CD158i (27.0%), CD158a (8.1%), and CD158b (5.4%).

The next-generation sequencing was available for 32/47 (68.1%) patients, and the genes mutated in at least 2 patients are summarized in Figure 1. Of notice, *STAT3* (13/32, 40.6%) was the most frequently mutated gene, with a prevalence of variants at the SH2 domain as follows: Y640F was detected in 6 cases (50.0%), D661Y in 4 cases (33.3%), and S614R was found in 2 cases (16.7%). *TET2* mutations occurred in 6 of 32 (18.8%). No *STAT5B* mutation were observed in our cohort.

Treatment response and survival analysis of NK-LGLL patients

Overall, during a median follow-up of 56.2 months (range 6.7-112.3 months), more than half (27/47, 57.4%) of patients required treatment. In this context, anemia (23/27, 85.2%) was the most prevalent indication for treatment rather than neutropenia and recurrent infection (4/27, 14.8%). The median number of treatment lines was 1 (range 1-4). MTX-based therapy and CsA-based therapy were delivered in 51.9% (14/27) patients and 29.6% of patients, respectively, as a first-line treatment. Others received CTX, splenectomy, or chemotherapy. Response rates are detailed in Table 2. The overall response rates (ORR) and complete response rates (CRR) for patients treated with MTX-based regimens were comparable to those treated with CsA-based regimens (ORR: 85.7% vs 87.5%, $P = 1.000$; CRR: 50.0% vs 37.5%, $P = .675$). Additionally, 9 of 27 (33.3%) patients treated with second-line immunosuppressants showed that response rates were not statistically different from those treated as first-line therapy (ORR: 66.7% vs 87.0%, $P = .314$; CRR: 22.2% vs 47.8%, $P = .249$).

With a median follow up of 52.7 months (range 6.7-112.3), only 1 patient died from esophageal and gastric varices bleeding during follow up (Supplementary Figure S1). The 5-year OS was 95.2%. There was no significant difference in PFS for patients treated with MTX and CsA-based regimens (median PFS: not reached vs 26.3 months, HR = 0.70, 95%CI, 0.18-2.67, $P = .597$) in first-line treatment (Supplementary Figure S2).

NK-LGLL patients with different CD56 expressions have distinct clinical and biological features

Considering the different exhibitions of immunophenotypes in mature NK cells, we divided patients into the CD56 dim/

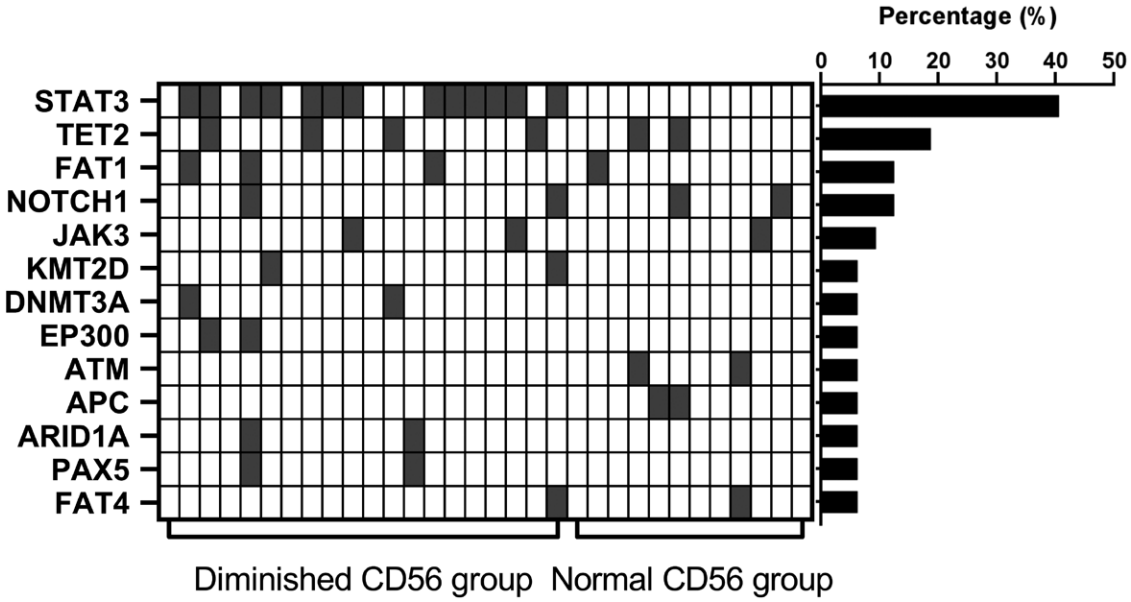

Figure 1. Gene mutation status in 32 NK-LGLL patients. This figure showed gene mutations observed at least in 2 patients which were detected by Next Generation Sequencing in 32 NK-LGLL. (1) *STAT3* was the most frequently mutated gene being consistent with other LGLL cohorts (40.6%). Ranking second, *TET2* mutation occurred in 6 of 32 cases (18.8%). Both *FAT1* and *NOTCH1* mutations were found in 4 cases (12.5%), with *JAK3* showing in 3 (9.4%). The remaining mutations were present in just 2 of 32 (6.3%) cases without exception. (2) Comparing the mutation frequency between the CD56 dim/ partial/ negative expression group and CD56 bright/moderate expression group, we could only find the difference in *STAT3*.

partial/negative expression group (28/47, 59.6%) and CD56 bright/moderate expression group (19/47, 40.4%) (Table 1), defined as diminished and normal CD56 groups respectively. Notably, we found that diminished CD56 patients displayed a skewed sex ratio of males (89.3% vs 31.6%, $P < .001$), and a more aggressive clinical features encompassing neutropenia (67.9% vs 0, $P < .001$), anemia (85.7% vs 15.8%, $P < .001$), splenomegaly (42.9% vs 10.5%, $P = .024$), and a higher necessity for therapeutic intervention (89.3% vs 15.8%, $P < .001$). Moreover, an elevated LDH (45.8% vs 11.1%, $P = .016$), and obvious fibrosis of bone marrow (92.3% vs 50.0%, $P = .006$) were also found in the diminished CD56 group.

Interestingly, the diminished CD56 group was characterized by a frequent occurrence of *STAT3* mutations, present in 61.9% of these cases, in contrast to none in the normal CD56 group ($P = .003$). Apart from the *STAT3* mutation, no significant difference in mutation frequency of other genes was observed between the 2 groups (Figure 1).

In the aspect of immunophenotypes, patients with diminished CD56 displayed a higher frequency of CD161 expression (54.5% vs 0.0%, $P < .001$). Despite this variations, CD8, CD16, CD57, CD94, Perforin, GramB, and KIRs expressions did not differ significantly between the groups. Moreover, in a subset of 14 patients from whom sequential samples were obtained, continuous analysis revealed consistent expression patterns. One individual with normal CD56 expression remained asymptomatic over a 12.8-month observation period, maintaining CD56 expression on NK-LGLs. Likewise, among those in the diminished CD56 group, CD56 expression levels persisted during treatment irrespective of the disease outcome, whether in remission (6/13) or progression (9/13).

NK-LGLL patients with diminished CD56 expression manifest a more aggressive clinical course

Among patients without indications for treatment at diagnosis, 1 of the 4 initially untreated patients in the diminished CD56 group commenced therapy due to moderate anemia after 48 months of observation. While none of the 13 patients in the normal CD56 group showed indications for treatment during follow up. The 4-year TTFT was shorter in diminished CD56 group compared to normal CD56 group without significance, given the limited number of terminal events (66.7% vs 100.0%, HR = 0.02, 95%CI, 0.0001-1.69, $P = .083$) (Figure 2). Furthermore, among the 23 patients treated with first-line immunosuppressors, the patients with diminished CD56 expression showed shorter PFS although without significant difference compared to normal CD56 expression group (median PFS: 26.3 months vs not reached, HR = 0.267, 95%CI, 0.05-1.36, $P = .112$) (Figure 3).

Discussion

In this comprehensive description of the large cohort of NK-LGLL patients, we established that CD56 servers as a crucial prognostic marker in NK-LGLL, signifying the necessity for treatment, as diminished CD56 expression correlates with a more symptomatic clinical manifestation. Moreover,

Table 2. Treatment response of NK-LGLL patients under different therapies.

Treatment	The response rate to first-line therapy, *n*/*N* (%)		The response rate to second-line therapy, *n*/*N* (%)	
	ORR	CRR	ORR	CRR
Immunosuppressor-based therapy	20/23 (87.0%)	11/23 (47.8%)	6/9 (66.7%)	2/9 (22.2%)
MTX-based	12/14 (85.7%)	7/14 (50.0%)	0/2 (0.0%)	0/2 (0.0%)
CsA-based	7/8 (87.5%)	3/8 (37.5%)	5/5 (100.0%)	2/5 (40.0%)
CTX-based	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)	0/1 (0.0%)
Other therapies	3/4 (75.0%)	3/4 (75.0%)		

Abbreviations: CRR, complete response rates; Csa, cyclosporine; CTX, cyclophosphamide; MTX, methotrexate; ORR, overall response rates.

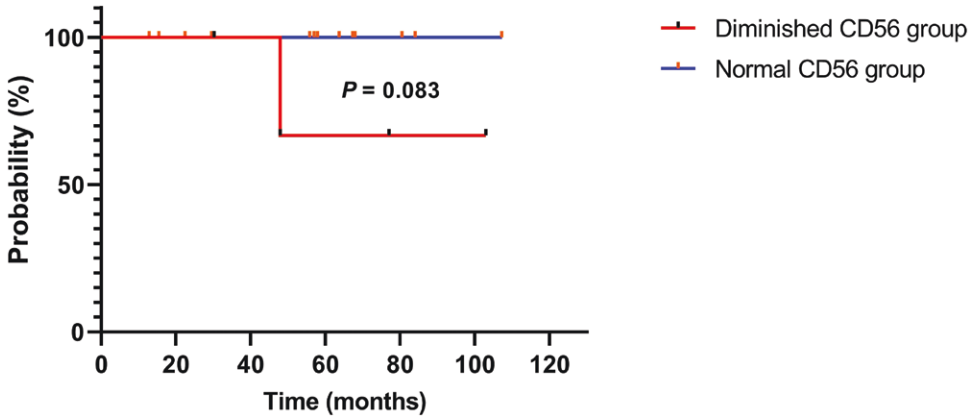

Figure 2. TTFT analysis of NK-LGLL patients between diminished and normal CD56 groups.

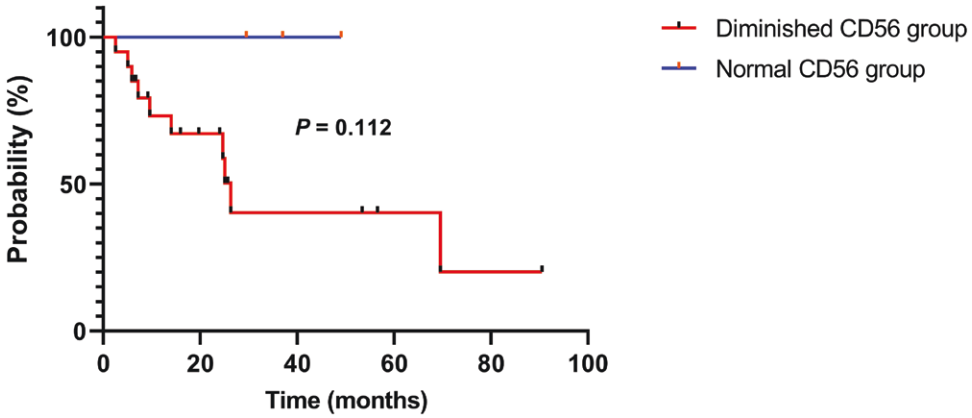

Figure 3. PFS analysis of NK-LGLL patients treated with immunosuppressors in first-line between diminished and normal CD56 groups.

the exclusive presence of *STAT3* mutations, CD161 expression, and an elevated degree of bone marrow fibrosis suggests a potentially distinctive pathogenesis in these patients, which may guide individual therapy.

Our results confirm and extend the characteristics of the NK-LGLL cohorts in other centers. Similarly, NK-LGLL was prevalent in the elderly, slightly inclined to male, and showed indolent course.[3] Contrasting to neutropenia in Western cohorts,[2,3,19] anemia was the most prevalent symptom in our study and occurred in close to 2/3 of the patients with a substantial portion presenting with more severe anemia and PRCA. Additionally, the predominant KIR phenotypes identified were either a complete absence of KIRs or a restrictive expression of activating KIRs and downregulation of inhibitory forms or lack of KIR expression. Our analysis highlights the significant role of KIR patterns in assessing NK-LGLL clonality, which not only suggests the clonality but also indicates the active phenotype of NK-LGL in disease conditions.[9,20,21]

To date, most data on treatment response is derived from discrete retrospective analyses, as immunosuppressive regimens are widely recognized as the first-line treatments for LGLL, owing to their favorable response rates.[14,18] Compared to the largest NK-LGLL cohorts, remarkably more patients achieved disease remission in our center (ORR: 54% vs 86%; CRR: 18% vs 50%). This discrepancy may be attributed to the fact that the most prevalent indication for treatment in our cohort was anemia, as LGLL patients with isolated anemia may achieve a great response according to the previous study.[22] Besides, nearly half of the patients were administered a combination of immunosuppressants and thalidomide, the latter being a classical immunomodulatory drug that has demonstrated favorable efficacy in our previous studies.[23] Moreover, it appears that treatment-naïve patients responded more favorably than those with relapsed or refractory disease, despite no statistically significant difference detected between initial and subsequent immunosuppression therapies due to the limited number of cases, which contrasts with the other large LGLL cohort.[24] Therefore, there is a pressing need for more prospective, multicenter clinical trials to thoroughly evaluate treatment responses and survival outcomes in LGLL, ensuring more reliable data to guide clinical decisions.

NK-LGLL is generally regarded as a chronic and indolent disease, while the differences among NK-LGLL patients remained ambiguous. Morice and Barila et al have previously reported that dim or negative CD56 expression in patients with NK-LGLL at diagnosis was associated with more pronounced clinical symptoms,[25,26] even in Tγδ LGLL.[7,27] To further elucidate internal heterogeneity of NK-LGLL, we stratified patients into 2 subsets based on CD56 expression levels. Align with prior research, patients in the diminished CD56 group presented with more severe neutropenia, anemia, and splenomegaly than the normal CD56 group, rendering them more responsive to treatment. During observation or first-line treatment period, patients in diminished CD56 group were prone to experience disease progression, whereas no patients in the normal CD56 group did, suggesting a more indolent course of patients with NK-LGLL with normal CD56 expression, even though no significant differences were shown in first-line PFS and TTNT for limited sample size and follow-up time. Additionally, CD56 diminished group was marked by CD161 expression, STAT3 mutations, and severe bone marrow fibrosis, implying a potentially distinct pathogenesis, which will be discussed further below. During the observation or first-line treatment period, patients in diminished CD56 group were prone to experience disease progression, whereas no patients in the normal CD56 group did, suggesting a more indolent course of NK-LGLL patients with normal CD56 expression. To sum up, these findings underscore the pivotal role of CD56 as a key marker for distinguishing disease severity and progression in NK-LGLL.

The distinctive biological features of diminished CD56 NK-LGLL patients may connote the reason why they present more aggressive symptoms. First, the cross-link of CD161 and CD16 activates NK cells enhances cytotoxicity and IFNγ production of NK-LGLs.[28] Second, while the direct impact of LGLs infiltration in the bone marrow on cytopenia remains debating,[29] the collagen deposition, which can impede the growth of hematopoietic stem cells, is a recognized factor exacerbating this condition.[30] Thirdly, *STAT3* mutation, which exclusively occurred in the diminished CD56 group, is a crucial indicator of several clinical challenges in previous research including neutropenia, anemia, thrombocytopenia, concurrent autoimmune diseases, greater need for treatment, sensitivity to immunosuppressive therapies, and reduced OS.[2,3,31-33] Pathogenically, on the one hand, *STAT3* mutation is commonly linked to cytotoxic immunophenotypes characterized by the upregulation of CD16, NKG2D, and the downregulation of NKG2A or inhibitory KIRs.[7,26,27,34] Meanwhile, patients accompanied by neutropenia were found

overexpression of MICA, the ligand of NKG2D, on granulocytes which boosts NK cell activation.[35] Both mechanisms underline that *STAT3* mutation would improve the cytotoxicity of LGLs, leading to cytopenia. On the other hand, *STAT3* mutation induces hypermethylation of miR-146b promoter which subsequently upregulates Fas ligand on LGLs and contributes to neutropenia through Fas-induced apoptosis.[36] However, in these cohort, the expression of activating KIRs, CD158i, did not differ between the 2 groups. Hence, in the future, we are going to screen a broader array of NK cell receptors and ligands, including KIRs and natural cytotoxicity receptor. By this, it will enhance our understanding of the relationship between specific immunophenotypes of NK-LGLs and their clinical manifestations, potentially guiding more therapeutic strategies.

In our study, NGS testing revealed that gene abnormalities of NK-LGLL predominantly involved in JAK/STAT pathway (*JAK3*), epigenomic regulation (*TET2*, *KMT2D*, *DNMT3A*, *EP300*), and various cancer-related genes (*FAT1*, *NOTCH1*, *ATM*, *ARID1A*, *APC*, *PAX5*, *FAT4*). Furthermore, *STAT3* was the most common mutation in about 40% of patients, and *TET2* was the second most frequent mutation observed, aligning with prior research.[33,37] *TET2* mutation frequency was similar between the 2 groups, and no distinction in immunophenotype and symptoms was found between patients with or without *TET2* mutation (not shown in results). However, in 2 NK-LGLL cohorts containing 46 and 58 cases, respectively, NK-LGLL patients harboring *TET2* mutations displayed lower platelet counts and CD16low pattern, often coinciding with other hematologic malignancies and showing resistant to immunosuppressive therapies.[33,37] Besides, research by Constance and Shunsuke et al identified *CCL22* mutated in 21% of NK-LGLL patients, who typically exhibited mild symptoms and a CD56bright/CD16dim immunophenotype.[38] Regrettably, the *CCL22* gene was not included in our NGS panel.

In conclusion, our findings indicate an indolent course and excellent response to immunosuppressive therapies of NK-LGLL. To further we classify NK-LGLL by CD56 and highlight that diminished expression of CD56 correlates with a unique cluster of biological features, more symptomatic disease, and a more aggressive disease course. This research contributes significantly to the classification and deeper understanding of NK-LGLL, paving the way for more refined treatment strategies and a deeper exploration of the underlying mechanisms driving this complex disease.

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Author contributions

Shuhua Yi conceptualized the study design. Yuxi Li, Rui Cui, and Ying Yu analyzed the data, performed statistical analyses, Yuxi Li wrote the manuscript. Yuxi Li, Rui Cui, Ying Yu, Yanshan Huang, Jingwen Sun, Jingjing Yuan, Tingyu Wang, Rui Lyu, Qi Wang, Wei Liu, Gang An , Weiwei Sui, Yan Xu, Wenyang Huang, Dehui Zou, and Huijun Wang acquired the data and managed the patients. Fengkui Zhang, Lugui Qiu,, and Shuhua Yi revised the manuscript critically and approved the final version.

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Conflict of interest

The authors declare no competing interests.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Supplementary material

Supplementary material is available at *The Oncologist* online.

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