Presence of serum antinuclear antibodies correlating unfavorable overall survival in patients with chronic lymphocytic leukemia

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Abstract

Background: Serum antinuclear antibodies (ANAs) are positive in some patients with chronic lymphocytic leukemia (CLL), but the prognostic value of ANAs remains unknown. The aim of this study was to evaluate the role of ANAs as a prognostic factor in CLL.

Methods: This study retrospectively analyzed clinical data from 216 newly diagnosed CLL subjects with ANAs test from 2007 to 2017. Multivariate Cox regression analyses were used to screen the independent prognostic factors related to time to first treatment (TTFT), progression free survival (PFS) and overall survival (OS). Receiver operator characteristic curves and area under the curve (AUC) were utilized to assess the predictive accuracy of ANAs together with other independent factors for OS.

Results: The incidence of ANAs abnormality at diagnosis was 13.9%. ANAs positivity and *TP53* disruption were independent prognostic indicators for OS. The AUC of positive ANAs together with *TP53* disruption was 0.766 (95% confidence interval [CI]: 0.697–0.826), which was significantly larger than that of either *TP53* disruption (AUC: 0.706, 95% CI: 0.634–0.772, $P = 0.034$) or positive ANAs (AUC: 0.595, 95% CI: 0.520–0.668, $P < 0.001$) in OS prediction. Besides, serum positive ANAs as one additional parameter to CLL-international prognostic index (IPI) obtained superior AUCs in predicting CLL OS than CLL-IPI alone.

Conclusion: This study identified ANAs as an independent prognostic factor for CLL, and further investigations are needed to validate this finding.

Keywords: Chronic lymphocytic leukemia; Antinuclear antibody; Autoimmunity; Prognosis; Overall survival

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by progressive accumulation of small, mature lymphocytes in the peripheral blood, bone marrow, and lymphoid tissues. Although considered to be rare in East Asia (CLL incidence of predominately Han Chinese was reported to be 0.05/100,000 per year),[1] increasing number of CLL cases has been identified in recent years. The clinical course of patients with CLL is highly heterogeneous, with some patients dying within rather short time, while others can even have a normal lifespan without any therapy. Therefore, investigators have made a great effort to elucidate the heterogeneity of CLL, and have found many prognostic factors to predict the outcome of this disorder. These prognostic factors include serum markers like beta-2 microglobulin (β2-MG), genetic markers including immunoglobulin heavy chain variable region (*IGHV*) and *TP53* mutational status, cytogenetic abnormalities such as del (13q), del(11q), and del(17p), as well as CD38 and ZAP-70 (ζ chain associated protein kinase 70) expression level.[2] More recently, use of next-generation sequencing has identified novel gene (eg, *NOTCH1*, *SF3B1*, *EGR2*, *NFKBIE*, and *FBXW7*) mutations,[3-6] which might be associated with aggressive clinical course and reduced survival.

Patients with CLL frequently present with immune disturbances. Common autoimmune diseases (AIDs) in CLL include autoimmune cytopenia such as autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), pure red cell aplasia (PRCA) and autoimmune granulocytopenia (AIG),[7-10] and non-hematological AIDs such as paraneoplastic pemphigus, neuropathies, Sjögren syndrome (SS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), etc.[11-13] The presence of AIHA was previously demonstrated to be a poor prognostic indicator,[8,14] and our center also proved the negative survival impact of positive direct antiglobulin test on CLL patients.[15] But the role of other non-hematological AIDs in CLL for prognosis is unclear.

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Antinuclear antibodies (ANAs) are directed against antigens of the cell nucleus. These autoantigens are named after their biochemical characteristics (deoxyribonucleic acid [DNA], histones, ribonucleoprotiens [RNP]), the disease associated with the corresponding autoantibody (Sjögren syndrome antigen A [SS-A, also known as Ro] and SS-B [also known as La]; polymyositis, progressive systemic sclerosis [PM-Scl]) or occasionally after the patient in whom the corresponding antibody was first detected (Sm, Ro, La).[16,17] ANAs can be used to diagnose different rheumatic diseases and judge disease activity, and are probably related to the pathogenesis of AIDs, such as anti-dsDNA and anti-Sm antibodies in SLE, rheumatoid factor (RF) in RA, etc.[18-21]

Recently, it was found that serum ANAs were positive in some CLL patients, but the prognostic value of ANAs remains unknown. This study aimed to evaluate the role of ANAs as a prognostic factor in CLL.

Methods

Ethical approval

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital. Informed written consent was obtained from all patients prior to their enrolment in this study.

Subjects

Two hundred and sixteen consecutive CLL patients were retrospectively enrolled from May 2007 to December 2017 at Department of Hematology, the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital. Baseline characteristics including gender, age, Binet stage, absolute lymphocyte count (ALC), hemoglobin (Hb), platelet (PLT), lactate dehydrogenase (LDH), albumin (ALB), β2-MG, and AIDs history were collected. Patients' survival data were further investigated to explore the prognostic value of ANAs. Diagnosis of CLL was based on criteria of the International Workshop on CLL-National Cancer Institute (IWCLL-NCI).[22] All the samples were collected prior to treatment.

Overall survival (OS) was defined as time from diagnosis until death. Time to first treatment (TTFT) was calculated as time from diagnosis to first treatment. Progression free survival (PFS) was referred to time from diagnosis until disease progression (PD). Patients without a documented event (for OS was death; for TTFT was start of CLL treatment; for PFS was disease progression) were censored at the date of last observation or February 26, 2018.

Cytogenetics

Fluorescence *in situ* hybridization (FISH) analysis was performed on most subjects to detect del(11q22.3) and del (17p13) using standard protocols as previously described.[23] The cut-off levels for positive values (mean of normal control ± 3 standard deviation [SD]), determined from samples of eight cytogenetically normal persons, was 7.7% and 5.2% for del(11q22.3) and del (17p13), respectively.

Detection of ZAP-70 and CD38

Flow cytometry was used to detect ZAP-70 and CD38 expression on fresh samples of CLL cells as previously described.[24] Data acquisition and analysis were performed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and Cell Quest software (BD Biosciences). Cut-off points of 30% and 20% were used to define positivity for CD38 and ZAP70, respectively.

Analyses of immunoglobulin heavy chain variable gene and TP53 mutations

IGHV sequencing was performed as described before,[25] and unmutated-*IGHV* (U-*IGHV*) was defined as ≥98% germline homology. Genomic DNA isolation and direct Sanger sequencing was performed for exon 4 to 9 of *TP53* as stated before.[26] This study referred the cohort with *TP53* mutation and/or del(17p13) as *TP53* disruptions.

Antinuclear antibody testing and antinuclear antibody profile (IgG) testing

ANAs in patients' sera were tested after diagnosis and before any treatment by indirect immunofluorescence test (IIFT), the gold standard for the determination of ANAs, with human epithelial (HEp-2) cells as substrate (EUROIMMUN, Lubeck, Germany). In this study, only patients with ANA titers of ≥1:100 were considered to be positive. Both of the sensitivity and specificity of the ANAs test are 100%, compared with enzyme-linked immuno sorbent assay (ELISA) and immunoblotting methods reported by the manufacturer. ANA profiles: anticentromere antibody (ACMA), anti-proliferating cell nuclear antigen (PCNA) antibody, nRNP/Sm, Sm, PO, SS-A, Ro-52, SS-B, Scl-70, Jo-1, PM-Scl, centromere protein-*B* (CENP-B), nucleosome, histones, anti-mitochondrial antibody (AMA)-M2, and ds-DNA (IgG) were further tested in ANAs positive patients using the EUROBLOT technology (EUROIMMUN). All the tests were performed in the Laboratory of Rheumatology, the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, with the recommended protocol by the manufacturer.

Statistical analysis

Categorical variables were compared using Chi-square test or Fisher exact test. Survival curves were plotted using Kaplan-Meier method and log-rank test was used for comparisons. Univariate and multivariate analyses were performed by Cox proportional-hazard regression, and hazards ratios (HR) were also calculated. Binary Logistic regression, receiver-operator characteristic (ROC) curve and corresponding area under the curve (AUC) were constructed to assess the predictive accuracy of positive ANAs together with other independent factors for OS or CLL-international prognostic index (IPI),[27] and the differences in AUCs were tested by a nonparametric

approach developed by DeLong *et al*.[28] Statistical analyses were performed using SPSS software version 21.0 (IBM Corporation, Armonk, NY, USA), Graphpad Prism 5 (GraphPad Software, San Diego, CA, USA), and MedCalc Software Version 18.2.1 (MedCalc Software, Ostend, Belgium). All tests were 2-sided, and statistical significance was defined as $P < 0.05$.

Results

Patients' baseline characteristics according to antinuclear antibody level

The characteristics of 216 newly diagnosed patients are listed in Table 1. One hundred and forty-one patients were males and 75 were females (male/female ratio: 1.88: 1), and the median age at diagnosis was 62 years (range: 16–86 years). When classified by Binet system, the number of each group was 85 (39.4%) in Binet A, 81 (37.5%) in Binet B, and 50 (23.1%) in Binet C. According to Rai stage system, 42 (19.4%) patients were classified into Rai stage 0, 110 (51.0%) patients were into Rai stage I–II, and 64 (29.6%) patients were into Rai stage III–IV.

In the 216 newly diagnosed CLL patients, positive ANAs were observed in 30 (13.9%) patients, which was much higher than that reported in normal people (5.6%–8.5%).[29-31] Eight of these 30 patients had ANA titers of 1: 320, 19 had ANA titers of 1:100, and 3 remain unavailable. These patients were further tested with antibodies to ANA profiles, and presented different expression levels of auto-antibodies (SS-A, Ro-52, SS-B, nucleosome, histones, ACMA, AMA-M2 and Sm, shown in Supplemental Table 1, http://links.lww.com/CM9/A15). Twelve patients were verified to be concomitant of AIDs, containing two ITP, one AIHA, three nephropathies confirmed by kidney biopsy (1 nephritis of Schonlein-Henoch purpura, one amyloidopathy, and 1 focal segmental glomerulosclerosis), 1 Hashimoto thyroiditis, 1 psoriatic arthritis, 1 Crohn disease and 1 patient suffered from both RA and SS. Only 5 of these 12 patients were with positive ANAs. Therefore, ANAs were presumed more associated with the disease of CLL.

The associations between ANAs and clinical and biological characteristics of CLL patients were also presented in Table 1. High levels of β2-MG ($P = 0.003$) and CD38 ($P = 0.027$) emerged as factors significantly related to the occurrence of serum ANAs. Serum presence of ANAs were not associated with gender, age, Binet stage, Rai stage, CLL-IPI, del(11q22.3), *TP53* and *IGHV* mutational status, ZAP-70 expression, treatment or other serum biomarkers (ALC, Hb, PLT, LDH, and albumin levels), indicating that distribution of covariates was adequately balanced and evenly distributed between the ANA positive and negative groups.

Patient treatments

Median follow-up time was 43 months (range: 2–129 months), during which 127 (58.8%) patients received treatment, and 89 (41.2%) patients did not receive any therapy due to not meeting the treatment indication.[32] The induction treatment included: (1) FC(M)R (fludarabine, cyclophosphamide, mitoxantrone, rituximab; $n = 32$, 25.2%); (2) F/FC/FCM (fludarabine-containing chemotherapy; $n = 13$, 10.2%); (3) bendamustine ($n = 17$, 13.4%); (4) bendamustine plus rituximab ($n = 1$, 0.8%); (5) chlorambucil and rituximab ($n = 3$, 2.4%); (6) chlorambucil ($n = 22$, 17.3%); (7) C(H)OP (cyclophosphamide, doxorubicin, vincristine, prednisone) or cyclophosphamide single drug ($n = 4$, 3.1%); (8) C(H)OP or EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin) plus rituximab ($n = 3$, 2.4%); (9) rituximab±fresh frozen plasma ($n = 17$, 13.4%); (10) ibrutinib ($n = 5$, 3.9%); (11) other regimens and supportive care ($n = 10$, 7.9%). We defined the above therapies numbered (1)–(4), (7), (8) and (10) as intensive treatments, and the remaining regimens as less intensive treatments. These strategies were chosen due to patients' age, performance status, and cytogenetics abnormalities.

Prognostic value of ANA in CLL

During the follow-up, 45 (20.8%) patients developed PD, and 36 (16.7%) patients passed away due to disease related death. In all of CLL patients, median TTFT was 35 months (range: 0–110 months), median PFS was 76 months (range: 0–110 months), and median OS was not reached (range: 0–115 months). The 5-year PFS rate was 67.1%, and 5-year OS rate was 73.7%. Ten of deaths (27.8%) owned positive ANAs, suggesting the potential prognostic value of ANAs.

We further studied the prognostic impact of ANAs on TTFT, PFS and OS. As demonstrated in Figure 1, the median TTFT for patients with positive ANAs was significantly shorter than those with negative ANAs (13 *vs*. 40 months, $P = 0.049$, Figure 1A). A similar result was also indicated as to OS with 54 months for ANAs positive group, while the median OS was not reached for ANAs negative group (54 months *vs*. not reached, $P = 0.017$, Figure 1C). However, ANA status showed no impact on PFS (56 *vs*. 75 months, $P = 0.988$, Figure 1B).

Next, we performed the univariate and multivariate analyses by Cox proportional-hazard regression to detect the independent prognostic factors related to TTFT, PFS, and OS of CLL. We included the classical factors like age, gender, Binet stages, ALC, Hb, PLT, del(11q22.3), *TP53* disruption status, *IGHV* mutational status, immunophenotyping markers (CD38 and ZAP-70), serological examinations (LDH, albumin, and β2-MG) as well as ANAs test [Table 2]. In univariate analyses, Binet stage B/C, ALC$\geq 50 \times 10^9$/L, Hb< 100 g/L, PLT$< 100 \times 10^9$/L, LDH>ULN, β2-MG>3.5 mg/L, *TP53* disruption, and unmutated-*IGHV* were significantly associated with shorter TTFT. Subsequently, these 8 parameters were included in multivariate Cox regression analyses. Finally, Binet stage B/C (HR: 2.190, 95% confidence interval [CI]: 1.415–3.389, $P < 0.001$), alongside with ALC$\geq 50 \times 10^9$/L (HR: 2.254, 95% CI: 1.434–3.540, $P < 0.001$) and β2-MG>3.5 mg/L (HR: 1.818, 95% CI: 1.220–2.709, $P = 0.003$) were independent predictors of shorter TTFT. As to PFS, Binet stage B/C, Hb< 100 g/L, PLT$< 100 \times 10^9$/L, LDH>ULN, β2-MG>3.5 mg/L, *TP53* disruption, and

Table 1: Clinical characteristics of CLL patients with or without positive ANAs.

Characteristics	Total (n=216)	Patients with positive ANAs (n=30)	Patients with negative ANAs (n=186)	χ^2 values	P
Gender				0.058	0.810
Male	141	19 (63.3)	122 (65.6)		
Female	75	11 (36.7)	64 (34.4)		
Age				0.355	0.552
≤65 years	133	17 (56.7)	116 (62.4)		
>65 years	83	13 (43.3)	70 (37.6)		
Rai Stage				2.087	0.352
0 (Low risk)	42	6 (20.0)	36 (19.3)		
I–II (Intermediate risk)	110	12 (40.0)	98 (52.7)		
III–IV (High risk)	64	12 (40.0)	52 (28.0)		
Binet Stage				1.277	0.259
A	85	9 (30.0)	76 (40.9)		
B/C	131	21 (70.0)	110 (59.1)		
CLL-IPI				2.854	0.091
0–3	131	14 (46.7)	117 (62.9)		
4–10	85	16 (53.3)	69 (37.1)		
ALC				0.079	0.778
$<50\times10^9$/L	176	25 (83.3)	151 (81.2)		
$\geq50\times10^9$/L	40	5 (16.7)	35 (18.8)		
Hb				3.878	0.091
<100 g/L	32	8 (26.7)	24 (12.9)		
≥100 g/L	184	22 (73.3)	162 (87.1)		
PLT				0.025	0.875
$<100\times10^9$/L	48	7 (23.3)	41 (22.0)		
$\geq100\times10^9$/L	168	23 (76.7)	145 (78.0)		
LDH*				1.338	0.247
≤ULN	175	22 (73.3)	153 (82.3)		
>ULN	41	8 (26.7)	33 (17.7)		
ALB				<0.001	0.991
<40g/L	79	11 (36.7)	68 (36.6)		
≥40g/L	137	19 (63.3)	118 (63.4)		
β2-MG				8.587	0.003
≤3.5 mg/L	116	10 (33.3)	106 (62.0)		
>3.5 mg/L	85	20 (66.7)	65 (38.0)		
TP53 disruption				0.365	0.546
Presence	35	6 (24.0)	29 (18.8)		
Absence	144	19 (76.0)	125 (81.2)		
Del (11q22.3)				3.769	0.107
Presence	24	6 (26.1)	18 (11.4)		
Absence	157	17 (73.9)	140 (88.6)		
IGHV gene				0.090	0.765
Unmutated	77	11 (45.8)	66 (42.6)		
Mutated	102	13 (54.2)	89 (57.4)		
CD38				4.890	0.027
<30%	145	15 (57.7)	130 (77.8)		
≥30%	48	11 (42.3)	37 (22.2)		
ZAP-70				0.875	0.350
<20%	105	16 (69.6)	89 (59.3)		
≥20%	68	7 (30.4)	61 (40.7)		
Treatments				0.338	0.561
Intensive treatments	76	15 (65.2)	61 (58.7)		
Less intensive treatments	51	8 (34.8)	43 (41.3)		

*The ULN of LDH in this study was 271 U/L. The data are shown as n or n (%). β2-MG: Beta-2 microglobulin; ALB: Albumin; ALC: Absolute lymphocyte count; ANA: Antinuclear antibody; CLL: Chronic lymphocytic leukemia; Hb: Hemoglobin; *IGHV*: Immunoglobulin heavy chain variable region; IPI: International Prognostic Index; LDH: Lactate dehydrogenase; PLT: Platelet; ULN: Upper limits of normal.

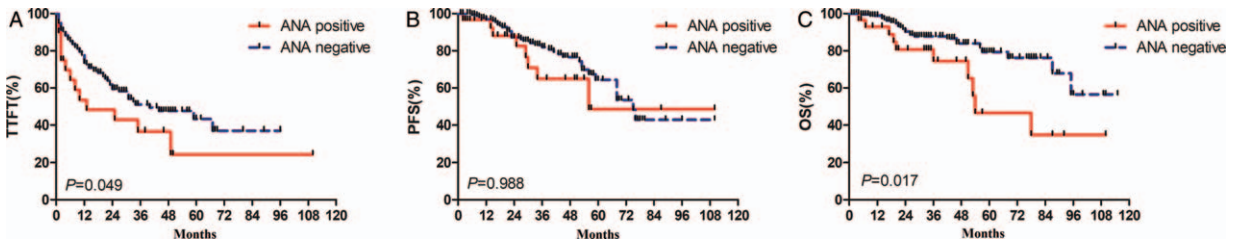

Figure 1: Kaplan-Meier curves of TTFT, PFS, and OS stratified by ANAs. (A) The median TTFT for patients with positive ANAs was significantly shorter than those with negative ANAs (13 *vs.* 40 months, $P=0.049$). (B) The median PFS for patients showed no difference between ANAs positive or negative groups (56 *vs.* 75 months, $P=0.988$). (C) The median OS for patients with positive ANAs was significantly shorter than those with negative ANAs (54 months *vs.* not reached, $P=0.017$). ANA: Antinuclear antibody; OS: Overall survival; PFS: Progression free survival; TTFT: Time to first treatment.

Table 2: Univariate and multivariate Cox regression analyses of prognostic factors for TTFT, PFS, and OS in this study.

	Univariate analyses		Multivariate analyses	
Variables	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
TTFT				
Binet B/C	2.819 (1.892–4.286)	<0.001	2.190 (1.415–3.389)	<0.001
ALC≥50×10^9/L	2.419 (1.628–3.594)	<0.001	2.254 (1.434–3.540)	<0.001
Hb<100 g/L	2.666 (1.734–4.101)	<0.001		
PLT<100×10^9/L	2.212 (1.502–3.260)	<0.001		
LDH>ULN*	1.740 (1.163–2.603)	0.007		
β2-MG>3.5 mg/L	2.256 (1.577–3.227)	<0.001	1.818 (1.220–2.709)	0.003
TP53 disruption	1.890 (1.216–2.937)	0.005		
Unmutated *IGHV*	1.616 (1.111–2.352)	0.012		
PFS				
Binet B/C	2.184 (1.061–4.496)	0.034		
Hb<100 g/L	3.527 (1.618–7.689)	0.002	3.618 (1.486–8.812)	0.005
PLT<100×10^9/L	3.344 (1.635–6.839)	0.001		
LDH>ULN*	2.982 (1.513–5.877)	0.002		
β2-MG>3.5 mg/L	3.840 (1.857–7.942)	<0.001		
TP53 disruption	4.598 (2.248–9.406)	<0.001	3.908 (1.730–8.830)	0.001
Unmutated *IGHV*	2.451 (1.218–4.930)	0.012	3.033 (1.444–6.372)	0.003
OS				
Hb<100 g/L	2.657 (1.234–5.721)	0.013		
β2-MG>3.5 mg/L	2.496 (1.218–5.114)	0.012		
TP53 disruption	6.685 (3.386–13.198)	<0.001	6.485 (3.285–12.801)	<0.001
ANAs positive	2.370 (1.140–4.927)	0.021	2.237 (1.058–4.729)	0.035

*The ULN of LDH in this study was 271 IU/L. β2-MG: Beta-2 microglobulin; ALC: Absolute lymphocyte count; CI: Confidence interval; Hb: Hemoglobin; HR: Hazards ratio; LDH: Lactate dehydrogenase; OS: Overall survival; PFS: Progression free survival; PLT: Platelet; TTFT: Time to first treatment; ULN: Upper limits of normal.

unmutated-*IGHV* were strongly associated with inferior PFS in univariate analyses. Hb<100 g/L (HR: 3.618, 95% CI: 1.486–8.812, $P=0.005$), *TP53* disruption (HR: 3.908, 95% CI: 1.730–8.830, $P=0.001$) and unmutated *IGHV* (HR: 3.033, 95% CI: 1.444–6.372, $P=0.001$) were independent PFS prognostic factors. Further, Hb<100 g/L, β2-MG>3.5 mg/L, *TP53* disruption, and positive ANAs were significantly correlated with shorter OS in univariate analyses. While *TP53* disruption (HR: 6.485, 95% CI: 3.285–12.801, $P<0.001$) and positive ANAs (HR: 2.237, 95% CI: 1.058–4.729, $P=0.035$) showed independent prognostic impact on OS in multivariate analyses. In conclusion, serum ANAs level was independent predictor for OS in CLL, but not for TTFT or PFS.

Binary Logistic regression and ROC curves were consequently constructed to analyze the ability of ANAs and *TP53* in predicting OS in CLL patients [Figure 2]. The AUC of positive ANAs together with *TP53* disruption was 0.766 (95% CI: 0.697–0.826), which was significantly superior to mere positive ANAs (AUC: 0.595, 95% CI: 0.520–0.668, $P<0.001$) or *TP53* disruption (AUC: 0.706, 95% CI: 0.634–0.772, $P=0.034$). Therefore, patients with disrupted *TP53* as well as positive ANAs might have a worse prognosis than those with either one of these factors.

Positive ANA improves the risk stratification of CLL-IPI

Since CLL is a heterogeneous disease, recently, a large-scale meta-analysis containing data from 3472 patients

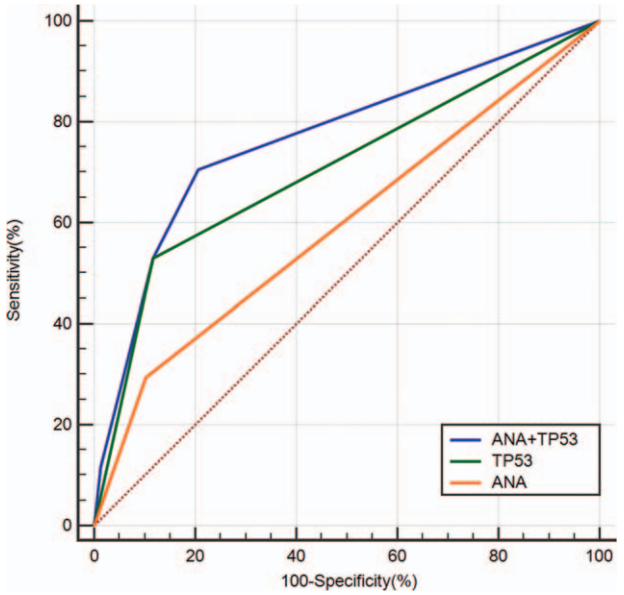

Figure 2: Positive ANAs improved the ability of *TP53* disruption in predicting OS in CLL patients. Curve of ANA plus *TP53*: AUC: 0.766, SE: 0.0450, 95% CI: 0.697–0.826; curve of *TP53* only: AUC: 0.706, SE: 0.0455, 95% CI: 0.634–0.772; curve of ANA only: AUC: 0.595, SE: 0.0416, 95% CI: 0.520–0.668 (ANA + *TP53 vs. TP53* only, $P = 0.034$; ANA + *TP53 vs.* ANA only, $P < 0.001$). ANA: Antinuclear antibody; AUC: Area under the curve; CI: Confidence interval; CLL: Chronic lymphocytic leukemia; OS: Overall survival; SE: Standard error.

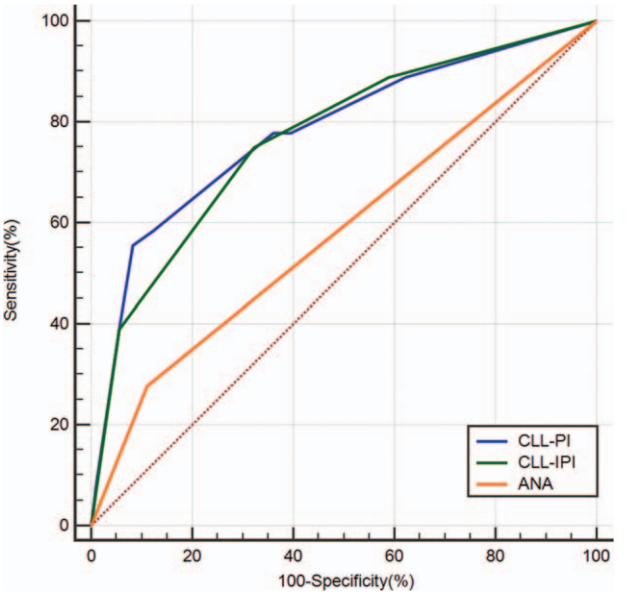

Figure 4: Positive ANAs improved the ability of CLL-IPI in predicting OS in CLL patients. Curve of CLL-PI (containing serum ANAs level): AUC: 0.781, SE: 0.0460, 95% CI: 0.720–0.835; curve of CLL-IPI: AUC: 0.769, SE: 0.0438, 95% CI: 0.707–0.824; curve of ANA only: AUC: 0.583, SE: 0.0396, 95% CI: 0.515–0.650 (CLL-PI *vs.* CLL-IPI: $P = 0.431$; CLL-PI *vs.* ANA only: $P < 0.001$). ANA: Antinuclear antibody; AUC: Area under the curve; CI: Confidence interval; CLL: Chronic lymphocytic leukemia; IPI: International prognostic index; OS: Overall survival; PI: Prognostic index; SE: Standard error.

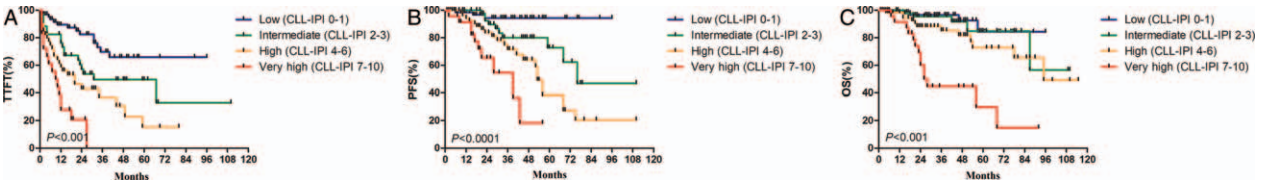

Figure 3: Kaplan-Meier curves of TTFT, PFS, and OS stratified by 4 CLL-IPI risk grades. CLL-IPI: Chronic lymphocytic leukemia-international prognostic index; OS: Overall survival; PFS: Progression free survival; TTFT: Time to first treatment.

proposed a new international prognostic index for CLL (CLL-IPI) to replace traditional Rai and Binet staging system.[27] Five independent prognostic factors were included in the CLL-IPI: *TP53* status, *IGHV* mutational status, serum β2-MG concentration (≤3.5 *vs.* >3.5 mg/L), clinical stage (Binet A or Rai 0 *vs.* Binet B–C or Rai I–IV), and age (≤65 *vs.* >65 years). Thus, we next used our data of Chinese CLL patients to validate CLL-IPI.

As is shown in Figure 3, the survival curves of TTFT and PFS were significantly distinguished in the 4 subgroups [Figure 3A and 3B], while the curves of OS in low-, intermediate- and high-risk subgroups were not well stratified (low *vs.* intermediate: $P = 0.616$; intermediate *vs.* high: $P = 0.221$; low *vs.* high: $P = 0.078$, Figure 3C). Given that serum ANAs, together with *TP53* status were independent prognostic factor for OS in the multivariate analyses, we proposed that combination of positive ANAs and CLL-IPI (which contains *TP53* status) might improve the prognostic capacity of CLL-IPI. We thus defined a novel irognostic index (CLL-PI) by the sum of CLL-IPI and one point for the presence of serum ANA. We discovered that positive ANA as 1 additional point to CLL-IPI obtained larger AUCs in OS prediction than CLL-IPI alone, although the difference was not statistically significant (AUC: 0.781 *vs.* 0.769, $P = 0.431$) [Figure 4].

Further validation of CLL-PI was conducted by segregating the CLL cohort into four risk grades: low-risk group (CLL-PI score: 0–2), intermediate-risk group (CLL-PI score: 3–5), high-risk group (CLL-PI score: 6–8) and very-high-risk group (CLL-PI score: 9–11). And pairwise comparisons for CLL-PI demonstrated more obvious differences in OS between each two specific groups (low *vs.* intermediate: $P = 0.090$, intermediate *vs.* high: $P = 0.039$, low *vs.* high: $P = 0.001$, high *vs.* very high: $P = 0.063$, Figure 5C). While the similar result was not reproducible for TTFT or PFS, due to ANA not being an independent prognostic factor for TTFT [Figure 5A] or PFS [Figure 5B]. Collectively, positive ANA may improve the risk stratification of CLL-IPI in OS prediction, and larger cohort should be included to validate this finding.

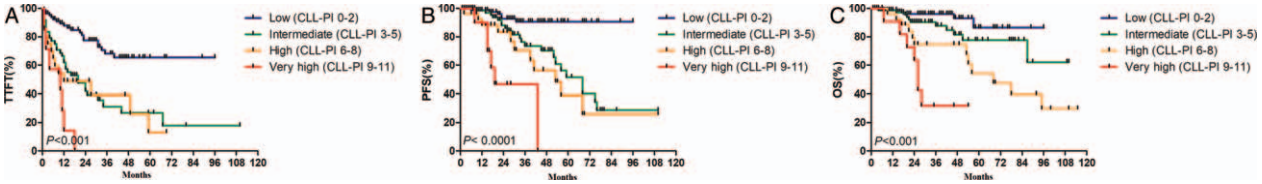

Figure 5: Kaplan-Meier curves of TTFT, PFS, and OS stratified by 4 CLL-PI risk grades. CLL: Chronic lymphocytic leukemia; OS: Overall survival; PFS: Progression free survival; PI: Prognostic index; TTFT: time to first treatment.

Discussion

ANAs have been well-described to be associated with diagnosis and prognosis in many rheumatic diseases, including SLE and RA. To our knowledge, this is the first study providing evidence of ANAs as a strong predictive marker for adverse prognosis in CLL survival. Although the pathophysiologic correlation between positive ANAs and malignancies is not fully elucidated, serum ANAs as a simple and easily measured parameter may be a good candidate for predicting prognosis in future clinical practice of CLL.

CLL patients are often accompanied with autoimmunity. Decades ago, CLL cells were found to produce antibodies (mostly IgM) against natural autoantigens such as nuclear antigens (ssDNA, dsDNA, histone proteins), as well as the Fc of IgG, vimentin, cardiolipin, cytoskeletal components, and RFs.[33-35] The property of them revealed high degree of multispecificity, binding to a number of different antigens. Besides, Hamblin *et al*[36] verified that non-hematological autoantibodies were positive in 21.5% (42/195) CLL patients. More recently, a GIMEMA study containing 3, 150 CLL patients by Barcellini *et al*[9] showed that 41% (93/227) of CLL patients had at least 1 positive test for a marker of autoimmunity, including 36 ANA, 25 ACA, 23 RF, 23 anti-TPO/anti-TG, 20 SMA, and 10 miscellaneous (AMA, anti-DNA, anti-PCA), and the incidence of ANA was 15.9%, which is consistent with our results. Barcellini *et al*[9] also found that serological autoantibodies and non-hematological AIDs were mostly observed in stage A patients, while hematologic complications (AIHA and ITP) were more easily occurred in patients with advanced disease (B+C stage). However, serum ANAs did not show any preference in early Binet stage in our study. Barcellini *et al*[9] suggested different pathogenic mechanisms underlying hematologic and non-hematological autoimmune phenomena in CLL but did not further study the prognostic value of autoantibodies, especially ANAs.

The source of ANAs in CLL patients is still an unanswered question. Some investigators demonstrated that autoantibodies are produced by the neoplastic B cells, because the autoantibodies secreted by the patients' B cells display monotypic Ig light chains that are identical to that expressed on the surface of the $CD5^+$ leukemic cells,[33-35] while others have suggested that they are produced by resting normal B cells as a consequence of T cell disturbance.[37] Further gene expression profiling (GEP) of CLL cells shows that these autoantibody-producing cells' expression profile does not resemble $CD5^+$ B cells, rather it has the features of memory B cells.[38] Therefore, the origins of ANAs are proposed as follows. Firstly, CLL cells may act as efficient antigen presenting cells inducing a T-cell response that, in turn, induces the subsequent activation of resting normal B cells and the production of polyclonal autoantibodies.[39] Secondly, CLL cells may also secrete inhibitory cytokines that alter immune tolerance, further facilitating the escape of self-reactive clones. Thirdly, in seldom cases, CLL cells may act as effector cells secreting a pathological monoclonal autoantibody.

The prognostic value of positive ANAs in CLL patients may be explained by autoantigens interaction with B cell receptors (BCRs). This was presumed according to the following reasons. First of all, the prognostic importance of the mutational status of *IGHV* genes indicates that CLL BCRs encounter antigens, which ultimately promote a certain degree of somatic hypermutations, which in turn influences the clinical behavior of the disease. In addition, the expression of quasi-identical ("stereotyped") BCRs among different patients with CLL suggests that a set of common antigens contributed to the stereotypy of the BCR in individual patients,[40-42] and this selective antigenic pressure may have a relevant role in the pathogenesis of the disease. In consistent with above statement, BCRs from *IGHV* unmutated cells are demonstrated to have low-affinity binding to a broader range of self-antigens,[43-45] whereas affinity-matured BCRs from *IGHV* mutated cases have high-affinity binding to restricted, more specific antigens. Last but not least, GEP studies demonstrate that BCR signaling is the key regulatory pathway activated in CLL cells in lymph nodes, the sites of CLL cell proliferation.[46,47] The activation of BCR is clarified to begin with antigens binding to the sIg of BCR and induce downstream pathways, further inducing CLL cells proliferation and migration.[48] Thus, we inferred that these antinuclear antigens might activate BCR and further stimulate CLL cells in this ligand dependent BCR signaling mechanism, and finally cause poorer OS in patients with positive ANAs. Of course, the pathogenesis of CLL is much more complex, resulting from the variety of underlying genetic lesions, degree of clonal evolution, epigenetic changes, activated signaling pathways and interaction with the microenvironment within lymph nodes or in the bone marrow.[49]

We have now entered into an era of precision medicine, cytogenetics, and molecular biology examinations further deepened our knowledge of disease. Our study was not intended to replace *TP53* disruption or CLL-IPI in clinical practice, but serum ANAs function should be well-assessed and managed in CLL patient care.

There were several drawbacks of our study. First, this was a retrospective study with potential selection bias. Second, the follow-up was not sufficient enough due to relatively long clinical course of CLL patients. Third, the sample size of our research was not large enough, and patients' baseline characteristics were not perfectly matched. However, we did Chi-square test, multivariate survival analyses and ROC curves to minimize these biases. Finally, dynamic changes of ANAs titers were not observed, especially in progressed or relapsed patients. Thus, further studies should include these data.

To conclude, positive ANAs was correlated with worse TTFT and OS in CLL patients and it was an independent prognostic factor for OS. Positive ANAs could improve the prognostic capacity of *TP53* disruption or CLL-IPI in OS prediction, which could be easily measured in all diagnostic laboratories. Future rigorous longitudinal investigations with larger samples and longer follow-up periods would allow us to disentangle the role of ANAs as a predictor of inferior prognosis in the CLL population.

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Conflicts of interest

None.

References

1. Yang C, Zhang X. Incidence survey of leukemia in China. Chin Med Sci J 1991;6:65–70.
2. Rai KR, Jain P. Chronic lymphocytic leukemia (CLL)-Then and now. Am J Hematol 2016;91:330–340. doi: 10.1002/ajh.24282.
3. Quesada V, Conde L, Villamor N, Ordonez GR, Jares P, Bassaganyas L, *et al*. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. Nat Genet 2011;44:47–52. doi: 10.1038/ng.1032.
4. Landau DA, Tausch E, Taylor-Weiner AN, Stewart C, Reiter JG, Bahlo J, *et al*. Mutations driving CLL and their evolution in progression and relapse. Nature 2015;526:525–530. doi: 10.1038/nature15395.
5. Young E, Noerenberg D, Mansouri L, Ljungstrom V, Frick M, Sutton LA, *et al*. EGR2 mutations define a new clinically aggressive subgroup of chronic lymphocytic leukemia. Leukemia 2017;31:1547–1554. doi: 10.1038/leu.2016.359.
6. Nadeu F, Clot G, Delgado J, Martin-Garcia D, Baumann T, Salaverria I, *et al*. Clinical impact of the subclonal architecture and mutational complexity in chronic lymphocytic leukemia. Leukemia 2018;32:645–653. doi: 10.1038/leu.2017.291.
7. Moreno C, Hodgson K, Ferrer G, Elena M, Filella X, Pereira A, *et al*. Autoimmune cytopenia in chronic lymphocytic leukemia: prevalence, clinical associations, and prognostic significance. Blood 2010;116:4771–4776. doi: 10.1182/blood-2010-05-286500.
8. Shvidel L, Tadmor T, Braester A, Bairey O, Rahimi-Levene N, Herishanu Y, *et al*. Pathogenesis, prevalence, and prognostic significance of cytopenias in chronic lymphocytic leukemia (CLL): a retrospective comparative study of 213 patients from a national CLL database of 1,518 cases. Ann Hematol 2013;92:661–667. doi: 10.1007/s00277-012-1663-3.
9. Barcellini W, Capalbo S, Agostinelli RM, Mauro FR, Ambrosetti A, Calori R, *et al*. Relationship between autoimmune phenomena and disease stage and therapy in B-cell chronic lymphocytic leukemia. Haematologica 2006;91:1689–1692. doi: 10.1016/j.exphem.2006.08.004.
10. Zent CS, Ding W, Reinalda MS, Schwager SM, Hoyer JD, Bowen DA, *et al*. Autoimmune cytopenia in chronic lymphocytic leukemia/small lymphocytic lymphoma: changes in clinical presentation and prognosis. Leuk Lymphoma 2009;50:1261–1268. doi: 10.1080/10428190903026492.
11. Anhalt GJ. Paraneoplastic pemphigus. J Investig Dermatol Symp Proc 2004;9:29–33. doi: 10.1111/j.1087-0024.2004.00832.x.
12. Sands J, Tuscano JM. Geoepidemiology and autoimmune manifestations of lymphoproliferative disorders. Autoimmun Rev 2010;9:A335–A341. doi: 10.1016/j.autrev.2009.11.009.
13. Hodgson K, Ferrer G, Montserrat E, Moreno C. Chronic lymphocytic leukemia and autoimmunity: a systematic review. Haematologica 2011;96:752–761. doi: 10.3324/haematol.2010.036152.
14. Hansen MM. Chronic lymphocytic leukaemia. Clinical studies based on 189 cases followed for a long time. Scand J Haematol Suppl 1973;18:3–286.
15. Xu W, Li JY, Miao KR, Cao X, Liu Q, Fan L, *et al*. The negative prognostic significance of positive direct antiglobulin test in Chinese patients with chronic lymphocytic leukemia. Leuk Lymphoma 2009;50:1482–1487. doi: 10.1080/10428190903111930.
16. Al Attia HM, Al Ahmed YH, Chandani AU. Serological markers in Arabs with lupus nephritis. Lupus 1998;7:198–201. doi: 10.1191/096120398678920000.
17. James K, Carpenter AB, Cook L, Marchand R, Nakamura RM. Development of the antinuclear and anticytoplasmic antibody consensus panel by the Association of Medical Laboratory Immunologists. Clin Diagn Lab Immunol 2000;7:436–443. doi: 10.1128/CDLI.7.5.858-858.2000.
18. Floris A, Piga M, Cauli A, Mathieu A. Predictors of flares in Systemic Lupus Erythematosus: preventive therapeutic intervention based on serial anti-dsDNA antibodies assessment. Analysis of a monocentric cohort and literature review. Autoimmun Rev 2016;15:656–663. doi: 10.1016/j.autrev.2016.02.019.
19. Adinolfi A, Valentini E, Calabresi E, Tesei G, Signorini V, Barsotti S, *et al*. One year in review 2016: systemic lupus erythematosus. Clin Exp Rheumatol 2016;34:569–574.
20. Bai Y, Tong Y, Liu Y, Hu H. Self-dsDNA in the pathogenesis of systemic lupus erythematosus. Clin Exp Immunol 2018;191:1–10. doi: 10.1111/cei.13041.
21. Tan EM, Smolen JS. Historical observations contributing insights on etiopathogenesis of rheumatoid arthritis and role of rheumatoid factor. J Exp Med 2016;213:1937–1950. doi: 10.1084/jem.20160792.
22. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Dohner H, *et al*. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008;111:5446–5456. doi: 10.1182/blood-2007-06-093906.
23. Xu W, Li JY, Pan JL, Qiu HR, Shen YF, Li L, *et al*. Interphase fluorescence in situ hybridization detection of cytogenetic abnormalities in B-cell chronic lymphocytic leukemia. Int J Hematol 2007;85:430–436. doi: 10.1532/ijh97.a10602.
24. Wu YJ, Wang H, Liang JH, Miao Y, Liu L, Qiu HR, *et al*. Using the geometric mean fluorescence intensity index method to measure ZAP-70 expression in patients with chronic lymphocytic leukemia. Onco Targets Ther 2016;9:797–805. doi: 10.2147/ott.s94613.
25. Chen L, Zhang Y, Zheng W, Wu Y, Qiao C, Fan L, *et al*. Distinctive IgVH gene segments usage and mutation status in Chinese patients with chronic lymphocytic leukemia. Leuk Res 2008;32:1491–1498. doi: 10.1016/j.leukres.2008.02.005.
26. Xia Y, Fan L, Wang L, Gale RP, Wang M, Tian T, *et al*. Frequencies of SF3B1, NOTCH1, MYD88, BIRC3 and IGHV mutations and TP53 disruptions in Chinese with chronic lymphocytic leukemia: disparities with Europeans. Oncotarget 2015;6:5426–5434. doi: 10.18632/oncotarget.3101.
27. International CLL-IPI working group. An international prognostic index for patients with chronic lymphocytic leukaemia (CLL-IPI): a meta-analysis of individual patient data. Lancet Oncol 2016;17:779–790. doi: 10.1016/s1470-2045(16)30029-8.
28. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 1988;44:837–845. doi: 10.2307/2531595.
29. Guyomard S, Salles G, Coudurier M, Rousset H, Coiffier B, Bienvenu J, *et al*. Prevalence and pattern of antinuclear autoantibodies in 347 patients with non-Hodgkin's lymphoma. Br J Haematol 2003;123:90–99. doi: 10.1046/j.1365-2141.2003.04587.x.

30. Theander E, Jonsson R, Sjostrom B, Brokstad K, Olsson P, Henriksson G. Prediction of Sjogren's syndrome years before diagnosis and identification of patients with early onset and severe disease course by autoantibody profiling. Arthritis Rheumatol 2015;67:2427–2436. doi: 10.1002/art.39214.
31. Zou HY, Gu X, Yu WZ, Wang Z, Jiao M. Detection of serum antinuclear antibodies in lymphoma patients. Genet Mol Res 2015;14:16546–16552. doi: 10.4238/2015.December.11.1.
32. Zelenetz AD, Gordon LI, Wierda WG, Abramson JS, Advani RH, Andreadis CB, *et al*. Chronic lymphocytic leukemia/small lymphocytic lymphoma, version 1.2015. J Natl Compr Canc Netw 2015;13:326–362.
33. Broker BM, Klajman A, Youinou P, Jouquan J, Worman CP, Murphy J, *et al*. Chronic lymphocytic leukemic (CLL) cells secrete multispecific autoantibodies. J Autoimmun 1988;1:469–481. doi: 10.1016/0896-8411(88)90068-6.
34. Sthoeger ZM, Wakai M, Tse DB, Vinciguerra VP, Allen SL, Budman DR, *et al*. Production of autoantibodies by CD5-expressing B lymphocytes from patients with chronic lymphocytic leukemia. J Exp Med 1989;169:255–268. doi: 10.1084/jem.169.1.255.
35. Borche L, Lim A, Binet JL, Dighiero G. Evidence that chronic lymphocytic leukemia B lymphocytes are frequently committed to production of natural autoantibodies. Blood 1990;76:562–569.
36. Hamblin TJ, Oscier DG, Young BJ. Autoimmunity in chronic lymphocytic leukaemia. J Clin Pathol 1986;39:713–716. doi: 10.1136/jcp.39.7.713.
37. Kipps TJ, Carson DA. Autoantibodies in chronic lymphocytic leukemia and related systemic autoimmune diseases. Blood 1993;81:2475–2487.
38. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, *et al*. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med 2001;194:1625–1638. doi: 10.1084/jem.194.11.1625.
39. Hall AM, Vickers MA, McLeod E, Barker RN. Rh autoantigen presentation to helper T cells in chronic lymphocytic leukemia by malignant B cells. Blood 2005;105:2007–2015. doi: 10.1182/blood-2003-10-3563.
40. Vardi A, Agathangelidis A, Sutton LA, Ghia P, Rosenquist R, Stamatopoulos K. Immunogenetic studies of chronic lymphocytic leukemia: revelations and speculations about ontogeny and clinical evolution. Cancer Res 2014;74:4211–4216. doi: 10.1158/0008-5472.can-14-0630.
41. Hoogeboom R, Wormhoudt TA, Schipperus MR, Langerak AW, Dunn-Walters DK, Guikema JE, *et al*. A novel chronic lymphocytic leukemia subset expressing mutated IGHV3-7-encoded rheumatoid factor B-cell receptors that are functionally proficient. Leukemia 2013;27:738–740. doi: 10.1038/leu.2012.238.
42. Xochelli A, Baliakas P, Kavakiotis I, Agathangelidis A, Sutton LA, Minga E, *et al*. Chronic lymphocytic leukemia with mutated IGHV4-34 receptors: shared and distinct immunogenetic features and clinical outcomes. Clin Cancer Res 2017;23:5292–5301. doi: 10.1158/1078-0432.ccr-16-3100.
43. Young RM, Staudt LM. Targeting pathological B cell receptor signalling in lymphoid malignancies. Nat Rev Drug Discov 2013;12:229–243. doi: 10.1038/nrd3937.
44. Hoogeboom R, van Kessel KP, Hochstenbach F, Wormhoudt TA, Reinten RJ, Wagner K, *et al*. A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. J Exp Med 2013;210:59–70. doi: 10.1084/jem.20121801.
45. Burger JA, Montserrat E. Coming full circle: 70 years of chronic lymphocytic leukemia cell redistribution, from glucocorticoids to inhibitors of B-cell receptor signaling. Blood 2013;121:1501–1509. doi: 10.1182/blood-2012-08-452607.
46. Herishanu Y, Perez-Galan P, Liu D, Biancotto A, Pittaluga S, Vire B, *et al*. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. Blood 2011;117:563–574. doi: 10.1182/blood-2010-05-284984.
47. Pal Singh S, Dammeijer F, Hendriks RW. Role of Bruton's tyrosine kinase in B cells and malignancies. Mol Cancer 2018;17:57. doi: 10.1186/s12943-018-0779-z.
48. Ten Hacken E, Burger JA. Molecular pathways: targeting the microenvironment in chronic lymphocytic leukemia–focus on the B-cell receptor. Clin Cancer Res 2014;20:548–556. doi: 10.1158/1078-0432.ccr-13-0226.
49. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic leukaemia. Nat Rev Cancer 2016;16:145–162. doi: 10.1038/nrc.2016.8.

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