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# High levels of CD20 expression predict good prognosis in chronic lymphocytic leukemia

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**Heterogeneity of CD20 expression exists in chronic lymphocytic leukemia (CLL), therefore, we explored the prognostic significance of CD20 expression in Chinese patients with CLL. Multiparameter flow cytometry was used to detect the expression of CD20 in $CD5^+CD19^+$ cells. In 172 CLL patients, the median expression percent of CD20 was 97.82% (range, 0–100), and the median mean fluorescence intensity (MFI) of CD20 in CLL cells was 731.45 (range, 0.00–9071.90). The percentage of $CD20^+$ cells in the patient group with mutated variable region of immunoglobulin genes (IGHV) was higher than in the non-mutant IGHV group (mean, 92.1% *vs* 80.4%, $P < 0.001$). There were no differences in the MFI of $CD20^+$ cells in all prognostic factor groups. Representation of the data using a receiver operating characteristic plot reflected separation between the two IGHV groups, with an area under the curve of 0.661 (95% confidence interval, 0.569–0.753). At the cut-off value of 60.3% for percentage of CD20, the sensitivity and specificity were 90.00% and 38.46%, respectively. Patients whose percentage of CD20 antigen was above 60.3% had longer treatment-free survival (hazard ratio, 0.452; 95% confidence interval, 0.232–0.884, $P = 0.020$). Percentage and MFI of CD20 were the variables not associated with treatment-free survival by multivariate Cox regression analysis ($P < 0.05$). High level of CD20 expression in de novo CLL appears to be associated with a good prognosis. (*Cancer Sci* 2013; 104: 996–1001)**

Chronic lymphocytic leukemia (CLL), the most frequently occurring leukemia in adults in North America and Europe, is a heterogeneous disease with variable clinical presentation and evolution.[1] In recent years, the significant increase in understanding the pathogenesis of CLL has been translated into a biologically oriented assessment of prognosis. In particular, there has been major progress in the identification of both molecular and cellular markers that may predict disease progression.[2,3] Two major molecular subtypes can be distinguished, characterized by a high or low number of somatic hypermutations in the variable region of immunoglobulin genes (IGHV) that features in CLL. However, IGHV gene sequencing remains a demanding technique, so many studies have focused on the identification of alternative markers with similar prognostic significance, and whose expression is easier to investigate, such as CD38 and zeta-associated protein-70 (ZAP-70).[4,5]

A great deal of renewed interest in CD20 membrane protein has emerged since 1997, when the chimeric anti-CD20 mAb rituximab was approved for use in relapsed or refractory low-grade or follicular B-cell non-Hodgkin's lymphoma. Rituximab is commonly incorporated into CD20-positive B-cell lymphoma therapy, including CLL, to improve response and prognosis. The CD20 expression level might correlate with treatment outcome in CLL patients. Tam *et al.*[6] showed that CLL patients with trisomy 12 showed strong expression of CD20 and had a high rate of response to rituximab-based therapy. However, Hsi *et al.*[7] reported that CD20 expression was not significantly correlated with clinical factors, and CD20 expression was also not associated with overall survival (OS). The prognostic significance of CD20 expression in B-cell precursor acute lymphoblastic leukemia has been extensively studied in children and adults.[8–10] In childhood acute lymphoblastic leukemia (ALL), Borowitz *et al.*[8] found that high CD20 intensity correlated with poorer event-free survival. Thomas *et al.*[11] also showed that CD20 positivity (20% or greater positive ALL cells) was associated with worse disease-free survival and OS, an effect especially relevant in patients aged 30 years and younger. Importantly, the ALL patients in these clinical studies were treated with either conventional or intensive frontline chemotherapy regimens that did not include rituximab. Diffuse large B-cell lymphoma (DLBCL) cells generally express the pan B-cell marker CD20, which is an important target for treatment. In their study of CD20 levels at onset of disease in patients with DLBCL, Suzuki *et al.*[12] found that CD20 negative status showed significantly inferior OS and progression-free survival compared to patients who were CD20 normal.

To date, there is limited information on the prognostic value of CD20 in CLL; it is important to shed light on the real function of this B-lineage differentiation molecule. The aim of our study was to study the correlation between CD20 expression and the clinical–biological characteristics of CLL patients.

## Material and Methods

**Ethics statement.** All patients provided written informed consent before enrolment in this study. The study was approved by the Institutional Review Board of the Nanjing Medical University (Nanjing, China) and carried out according to the Declaration of Helsinki.

**Patients.** One hundred and seventy-two consecutive Chinese patients with CLL were enrolled from January 2003 and December 2011. Blood samples collected at diagnosis were available for CD20, ZAP-70, and CD38 analyses. The diagnosis of CLL was based on clinical characteristics, peripheral blood morphologies, immunophenotype, and B-lymphocytes $\geq 5.0 \times 10^9$/L.[13] Clinical stage was determined using the Binet staging system according to the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) criteria. Treatment of CLL was based on the IWCLL criteria. The chemotherapy regimens used included: fludarabine and cyclophosphamide; fludarabine, cyclophosphamide, and rituximab; high-dose methylprednisolone; rituximab and high-dose

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methylprednisolone; cyclophosphamide, vincristine, and prednisone; and chlorambucil.

**Cellular immunophenotypic analysis.** Flow cytometric analysis was carried out on fresh peripheral blood samples. All flow cytometric analyses were carried out on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument was aligned and calibrated daily with the use of a four-color mixture of CaliBRITE beads (BD Biosciences) with FACSComp Software according to the manufacturer's instructions. The beads were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity in order to monitor instrument performance over time. Whole blood was stained with the following three-color combination of mAbs, CD19/CD22/CD20, CD19/CD5/CD38, and CD19/Lambda/kappa, directly conjugated with phycoerythrin-cyanin 5.1 (PC5), phycoerythrin (PE), and FITC, respectively. Murine isotype control (BD Biosciences) conjugated with FITC, PE, and PC5 were applied with each sample to set the negative gate. ZAP-70-PE (Caltag Laboratories, Burlingame, CA, USA) was used to determine cytoplasmic ZAP-70 expression according to the manufacturer's instructions. Multicolor flow cytometry was carried out as previously described.[14,15] Briefly, fresh whole blood samples were incubated with a mixture of fluorescence-labeled mAbs for 15 min at room temperature in the dark. Lysis of mature red blood cells was done using Tris-NH4Cl solution. Data analysis was carried out with CellQuest software (BD Biosciences), cell subpopulations of interest were delineated using CD45/side scatter dot plots, and after subgating CD19-positive tumor cells, CD20 expression positivity and mean fluorescence intensity (MFI) were calculated. At least 10 000 events were measured from each sample. Cut-off points of 30% and 20% were used to define positivity for CD38 and ZAP-70, respectively.

**Detection of molecular cytogenetic aberrations by FISH.** The FISH analysis was carried out for conventional cytogenetic studies. Fluorescent-labeled probes (Vysis, Downers Grove, IL, USA) were used in interphase cytogenetic analyses to detect prognostically relevant abnormalities of chromosomal regions 6q23 (LSI MYB), 11q22.3 (LSI ATM), 13q14 (LSI D13S319), 14q32 (LSI IGHC/IGHV), 17p13 (LSI p53), and centromere 12 (CEP12). The analysis was carried out as previously described.[15] The cut-off levels for positive values (mean of normal control $\pm$ 3 SD), determined from samples of eight cytogenetically normal persons, was 7.5%, 7.7%, 10.3%, 8.9%, 5.2%, and 3.0% for del(6q23), del(11q22.3), del(13q14), 14q32 translocation, del(17p13), and trisomy 12, respectively.

**Analysis of p53 mutation and IGHV mutation status.** Genomic DNA was isolated from PBMC preparations stored at $-80$°C. Primers to amplify exons 2/3, 4, 5/6, 7, 8/9, 10, and 11 of the human *p53* gene and adjacent intronic sequences (GenBank accession NG_017013.1) were designed using the Primer 5 program (http://www.premierbiosoft.com). The PCR products were purified using standard methods (Invitrogen, Shanghai, China) and directly sequenced with primer p53 ex2-3-F, ex4-R, ex5-6-F, ex-7-F, ex8-9-F, ex-10-F, and ex-11-F using the ABI3730XL 96-capillary DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing of IGHV was carried out as previously described.[16] A germline homology of 98% was used as the cut-off between IGHV mutated and non-mutated cases.

**Statistical analysis.** All statistical analyses were carried out with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Group-wise comparisons of CD20 percentages, MFI, and the other biological and clinical variables were carried out, applying two-tailed Mann–Whitney *U*-tests and Kruskal–Wallis tests with Dunn multiple comparisons. Receiver operating characteristic curve (ROC) and area under the ROC curve (AUC) were established to verify the diagnostic value of CD20 in differentiating the mutation or non-mutation of IGHV. Treatment-free survival (TFS) was defined as the period from the diagnosis date to either the time of the first CLL-specific treatment or to the last follow-up date. Treatment-free survival was estimated according to the Kaplan–Meier method and compared between groups by means of the log–rank test. Cox's proportional hazards regression models were used to assess the independent effect of covariables on the TFS. A *P*-value of $<0.05$ was considered statistically significant, and all tests were two-tailed.

## Results

**Patient characteristics.** The baseline characteristics of the study participants are presented in Table 1. One hundred and thirteen patients were male, and 59 were female (male:female ratio, 1.92:1.00). The median age of the group overall was 62 years (range, 35–93 years); 62.79% were aged 60 or older. According to the Binet clinical staging system, 83 patients (48.26%) were classified as Binet stage A, 36 (20.93%) as Binet stage B, and 53 (30.81%) as Binet stage C. Flow cytometry analysis of peripheral blood specimen lymphocytes showed that 119 (70.41%) of 169 patients had <30% of lymphocytes expressing CD38; and 29.59% of patients had more than 30% CD38-positive lymphocytes. Forty-four patients (26.35%) had CLL cells that expressed ZAP-70 by flow cytometry, and 52 patients (33.98%) had CLL cells that expressed non-mutated IGHV. Mutation of the *p53* gene was present in 28 patients (17.95%). Nine patients (5.23%) had del (13q14) as the sole abnormality, 45 patients (26.16) had del (17p13) deletion or del(11q22.3). With a median follow-up period of 27 months (range, 1–97 months) from CLL diagnosis, eight patients died of CLL-related causes. Eighty-one of 172 patients (47.09%) received chemotherapy for their disease according to the IWCLL criteria. Eighteen patients (50%) were treated with rituximab chemotherapy in the treatment-naïve state. Eighteen patients were also treated with rituximab chemotherapy, but these patients were all in the relapsed state of disease.

**Expression of CD20 and association with other prognostic factors.** In a total of 172 CLL cases, the median expression percent of CD20 was 97.82% (range, 0–100%), and MFI of CD20 on CLL cells was 731.45 (range, 0–9071.90). The possibility of interaction between CD20 and other known prognostic factors was analyzed in our cohort (Table 2). There were no differences in the percentage of CD20-positive cells in the prognostic factors groups, such as Binet stage, ZAP-70, CD38 expression, *p53* mutation status, and cytogenetic abnormalities ($P > 0.05$), but expression of CD20 was associated with IGHV mutational status in CLL ($P < 0.001$). The percentage of CD20-positive cells in cases of mutated IGHV groups was higher than non-mutant groups (mean, 92.1% *vs* 80.4%). There were also no differences in the MFI of CD20-positive cells in each prognostic factors group ($P > 0.05$). Compared with non-mutant groups, MFI of CD20-positive cells in mutated IGHV groups was higher (mean, 1396 *vs* 1051.4), however, there was no statistical significance ($P = 0.19$)

**Analyses using ROC and AUC.** We carried out the ROC curve and AUC analyses to assess the sensitivity and specificity of the CD20 signature for CLL prognosis risk estimation. Representation of the data using a ROC plot reflected strong separation between these two subgroup of CLL with an AUC of 0.661 (95% confidence interval [CI], 0.569–0.753) for the percentage of CD20 (Fig. 1). At the cut-off value of 60.3% for percentage of CD20, the sensitivity and specificity were 90.00% and 38.46%, respectively.

**Prognostic impact of CD20 on TFS.** According to the set cut-off value of percentage of CD20, TFS was researched in the CD20


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**Table 1. Baseline characteristics of 172 Chinese patients with chronic lymphocytic leukemia ($n$ = 172)**

| Parameter | Median (range) | Cases (%) |
|---|---|---|
| Gender ($n$ = 172) | | |
| Male | | 113 (65.70) |
| Female | | 59 (34.30) |
| Age | 62 (35–93) | |
| ≥60 years | | 108 (62.79) |
| <60 years | | 64 (37.21) |
| Binet stage ($n$ = 172) | | |
| A | | 83 (48.26) |
| B | | 36 (20.93) |
| C | | 53 (30.81) |
| CD38 ($n$ = 169) | | |
| Positive | | 50 (29.59) |
| Negative | | 119 (70.41) |
| ZAP-70 ($n$ = 167) | | |
| Positive | | 44 (26.35) |
| Negative | | 123 (73.65) |
| IGHV ($n$ = 153) | | |
| Unmutated (≤2% deviation from a germline) | | 52 (33.98) |
| Mutated (>2% deviation from a germline) | | 101 (66.02) |
| *p53* Mutation status ($n$ = 156) | | |
| Presence | | 28 (17.95) |
| Absence | | 128 (82.05) |
| Treatment ($n$ = 172) | | |
| No | | 91 (52.91) |
| Yes | | 81 (47.09) |
| Treatment regime ($n$ = 81) | | |
| Rituximab-based regimes | | 36 (44.44) |
| Other regimes | | 45 (55.56) |
| Cytogenetics ($n$ = 172) | | |
| del(17p13) or del(11q22.3) | | 45 (26.16) |
| del(13q24) as the sole abnormality | | 9 (5.23) |
| Other | | 118 (68.60) |
| CD20% ($n$ = 172) | 97.82% (0–100) | |
| CD20 MFI ($n$ = 172) | 731.45 (0–9071.90) | |

IGHV, variable region of immunoglobulin genes; MFI, mean fluorescence intensity; ZAP-70, zeta-associated protein-70.

subgroup. Patients whose percentage of CD20 antigen was above 60.3% had a better TFS (hazard ratio [HR], 0.452; 95% CI, 0.232–0.884, $P$ = 0.020) (Fig. 2). Pretreatment characteristics that correlated with longer TFS by univariable analysis are shown in Table 3. Predictors for progression-free survival were ZAP-70 (≥20%) (HR = 1.959; 95% CI, 1.23–3.12, $P$ = 0.005) and IGHV mutation (HR = 0.478; 95% CI, 0.303–0.755, $P$ = 0.002). To assess whether CD20 percentage alone represents a prognostic factor, the group of patients who underwent rituximab chemotherapy were analyzed separately from the patients without rituximab. The rituximab treatment affected the prognostic impact of the percentage of CD20 in patients in a naïve state of disease ($P$ = 0.12; $P$ = 0.52) (Fig. S1), however, we cannot draw any conclusions from this data because of the limited number of patients. We researched the co-expression pattern of ZAP-70 and CD20 and their impact on TFS (Fig. S2). There were significant statistical differences between the groups ($P$ = 0.01) The low ZAP-70/high CD20 group had longer TFS when compared with the other groups, with median TFS times being 72 months (low ZAP-70/high CD20), 15 months (high ZAP-70/high CD20), 4 months (low ZAP-70/low CD20), and 3 months (high ZAP-70/low CD20), respectively. In multivariable analysis, no variable selection technique was used, and all variables remained in the multivariable model. Multivariate Cox proportional hazards models were used to study factors associated with TFS endpoints in CLL by both Enter and Forward conditional methods, pretreatment patient characteristics independently associated with lower risk of disease progression included Binet A, higher CD20 expression, lower ZAP-70 expression, and IGHV mutation (Table 4). Percentage of CD20 was not associated with TFS by multivariate Cox regression analysis ($P$ = 0.087). In conclusion, a higher level of CD20 expression in de novo CLL appears to be associated with a better prognosis.

## Discussion

Chronic lymphocytic leukemia is less effectively treated than other B-cell malignancies with the anti-CD20 agent rituximab, presumably, at least in part, due to low CD20 expression.[17] Expression of CD20 is typically measured by flow cytometry. The aim of our study was to study the correlated CD20 expression with clinical–biological characteristics.

Previously, there are few data on the level of CD20 expression in Chinese CLL patients. Data presented in this report confirm that total levels of CD20 protein are low in CLL B cells. The median expression percent of CD20 was 97.82% (range, 0–100%), and the median MFI of CD20 on CLL was 731.45 (range, 0.00–9071.90). Ginaldi *et al.*[18] first reported that the level of CD20 expression in patients with the leukemic form of B-cell lymphoma is significantly lowest in CLL and the highest in hairy cell leukemia. The number of CD20 molecules was lower in CLL than in normal B cells and the other B-cell leukemias ($P < 0.05$). CD20 is a transmembrane phosphoprotein that functions as a calcium channel, and it has been shown to play an important role in B cell activation and differentiation.[19] CD20 expression appears later than other B-cell markers during normal B lymphocyte development and its membrane density progressively increases during differentiation.[20] The low percentage and intensity of CD20 in CLL probably reflects an earlier stage of maturation of the B-CLL lymphocytes compared with normal blood B lymphocytes and other malignant B cells. The CLL clones use either mutated or non-mutated IGHV genes and this feature gave rise to the postulate that the two subgroups of CLL originated from distinct cell types. We found that lower CD20 expression was significantly correlated with non-mutation of IGHV. Low expression of CD20 CLL cells may originate from antigen-experienced B lymphocytes that have no IGHV gene mutation.

From a clinical viewpoint, numerous clinical and preclinical studies on rituximab indicate that treatment with this drug has outgrown its empirical use. We can confirm its efficacy only in patients in whom CD20 expression was determined in CLL cells, but we are not sure whether the level of surface CD20 expression and CD20 MFI could be a predictive factor, identifying the patients in whom treatment with rituximab would be efficient and those in whom the use of this drug would be inefficient. Johnson *et al.*[21] showed that CD20 expression and response to treatment with rituximab are associated in DLBCL patients. Patients with a level of CD20 expression above the cut-off value had a significantly longer OS and a significantly higher overall response rate than those patients with CD20 expression levels below the cut-off value. However, we observed no significant difference in TFS between the two groups, although there was a trend toward lower risk for achievement of treatment ($P$ = 0.087). The reason that we did not see any significant $P$-values for CD20 expression in either current model may be related to parameter and parameter interaction. As our study was


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**Table 2. Correlations between CD20 expression and disease features of chronic lymphocytic leukemia**

| Parameter | CD20% | | | CD20 MFI | | |
|---|---|---|---|---|---|---|
| | No. of cases | Median (*P*5–*P*95) | *P*-value | No. of cases | Median (*P*5–*P*95) | *P*-value |
| Gender (*n* = 172) | | | | | | |
| Male | 113 | 96.22 (37.26–100) | 0.30 | 113 | 863.44 (88.78–4451.36) | 0.20 |
| Female | 59 | 100 (51.10–100) | | 59 | 588.52 (81.05–3466.56) | |
| Age (*n* = 172) | | | | | | |
| ≥60 years | 109 | 97.71 (46.55–100) | 0.88 | 109 | 952.26 (99.05–3933.46) | 0.06 |
| <60 years | 63 | 98.40 (36.84–100) | | 63 | 524.15 (53.50–4477.11) | |
| Binet stage (*n* = 172) | | | | | | |
| A | 83 | 97.71 (53.70–100) | 0.52 | 83 | 731.64 (77.23–4630.52) | 0.48 |
| B | 36 | 91.90 (31.87–100) | | 36 | 547.28 (84.81–3250.45) | |
| C | 53 | 100 (27.60–100) | | 53 | 833.09 (159.80–4044.24) | |
| CD38 (*n* = 169) | | | | | | |
| Positive | 50 | 98.73 (56.09–100) | 1.00 | 50 | 733.38 (92.45–3933.07) | 0.60 |
| Negative | 119 | 97.71 (37.97–100) | | 119 | 731.64 (82.81–3915.94) | |
| ZAP-70 (*n* = 167) | | | | | | |
| Positive | 44 | 97.08 (52.40–100) | 0.86 | 44 | 598.21 (97.36–3935.30) | 0.84 |
| Negative | 123 | 97.46 (39.00–100) | | 123 | 751.56 (96.03–3857.34) | |
| IGHV (*n* = 153) | | | | | | |
| Unmutated | 52 | 90.48 (3.03–100) | <0.001 | 52 | 480.71 (78.74–3569.62) | 0.19 |
| Mutated | 101 | 100 (50.99–100) | | 101 | 751.56 (83.86–4666.35) | |
| p53 mutation status (*n* = 156) | | | | | | |
| Presence | 28 | 96.61 (3.97–100) | 0.36 | 28 | 469.89 (145.07–4737.80) | 0.73 |
| Absence | 128 | 98.29 (51.92–100) | | 128 | 731.45 (81.84–3933.82) | |
| Treatment (*n* = 172) | | | | | | |
| No | 91 | 100 (56.42–100) | 0.10 | 91 | 930.26 (79.14–4324.41) | 0.30 |
| Yes | 81 | 95.06 (37.56–100) | | 81 | 559.89 (107.62–3533.46) | |
| Cytogenetics (*n* = 172) | | | | | | |
| del(17p13) or del(11q22.3) | 45 | 98.96 (3.78–100) | 0.59 | 45 | 548.16 (93.49–6766.42) | 0.21 |
| del(13q14) as the sole abnormality | 9 | 88.83 (65.93–100) | | 9 | 313.30 (82.81–3199.19) | |
| Others | 118 | 97.82 (44.60–100) | | 118 | 818.77 (90.59–3930.09) | |

IGHV, variable region of immunoglobulin genes; MFI, mean fluorescence intensity; ZAP-70, zeta-associated protein-70.

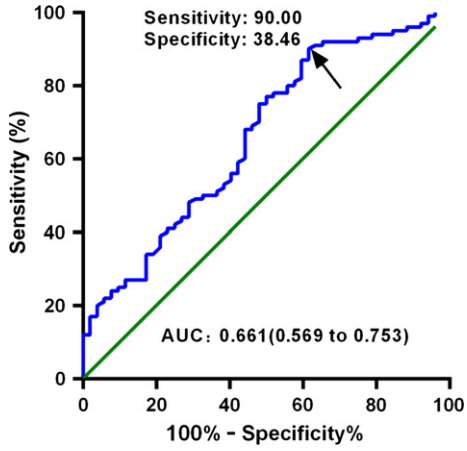
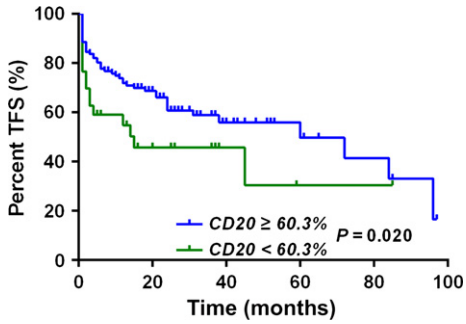



**Fig. 1.** Receiver operating characteristic curve of CD20 expression to predict the mutation status of the variable region of immunoglobulin genes in chronic lymphocytic leukemia. AUC, area under the curve. Arrows point to cutoff value of 60.3% percentage of CD20, the sensitivity and specificity were 90.00% and 38.46%, respectively.




| | Median (95% CI), months |
|---|---|
| CD20% ≥ 60.3 | 60 (25.56–94.44) |
| CD20% < 60.3 | 15 (0.00–25.63) |

**Fig. 2.** Prognostic impact of CD20 on treatment-free survival (TFS) in patients with chronic lymphocytic leukemia. Those patients whose percentage of CD20 antigen was above 60.3% had a better TFS (hazard ratio [HR], 0.452; 95% confidence interval [CI], 0.232–0.884, *P* = 0.020).

retrospective, both factors contributed to our results, therefore the study might not necessarily be representative.

The results of our study indicate that the level of CD20 expression is an important predictive factor. Datasets compiled by the prospective collection of well-characterized CLL patients are now surely warranted in order to confirm the clinical significance of CD20 in CLL.

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**Table 3. Univariable Cox proportional hazards models for treatment-free survival in Chinese patients with chronic lymphocytic leukemia ($n$ = 172)**

| Characteristic | No. of cases | Hazard ratio (95% CI) | *P*-value |
|---|---|---|---|
| Age (years), <60 *vs* ≥60 | 172 | 0.141 (0.894–2.197) | 0.1410 |
| Gender (male *vs* female) | 172 | 1.326 (0.825–2.133) | 0.2440 |
| Binet stage A (*vs* C) | 136 | 0.231 (0.131–0.405) | **<0.0001** |
| Binet stage B (*vs* C) | 89 | 0.979 (0.589–1.626) | 0.9340 |
| CD38 ≥ 30% (*vs* <30%) | 169 | 1.493 (0.948–2.354) | 0.0840 |
| ZAP-70 ≥ 20% (*vs* <20%) | 167 | 1.959 (1.230–3.120) | **0.0050** |
| IGHV mutation (*vs* non-mutation) | 153 | 0.478 (0.303–0.755) | **0.0020** |
| p53 mutation (*vs* non-mutation) | 156 | 1.507 (0.879–2.582) | 0.1360 |
| Unfavorable karyotype† (*vs* favorable‡) | 54 | 1.072 (0.457–2.513) | 0.8730 |
| Others (*vs* favorable‡) | 127 | 0.590 (0.261–1.335) | 0.2050 |
| CD20 ≥ 60.3% (*vs* <60.3%) | 172 | 0.452 (0.232–0.884) | **0.0200** |

†Unfavorable karyotype, del(17) and del(11q). ‡Favorable karyotype, del(13q24) as the sole abnormality. CI, confidence interval; IGHV, variable region of immunoglobulin genes; MFI, mean fluorescence intensity; ZAP-70, zeta-associated protein-70. Bold values indicate statistically significant.

**Table 4. Multivariate Cox proportional hazards models for treatment-free survival (TFS) in chronic lymphocytic leukemia**

| Characteristic | Hazard ratio (95% CI) | *P*-value |
|---|---|---|
| TFS ($n$ = 138) | | |
| Age (years), <60 *vs* ≥60 | 1.117 (0.661–1.885) | 0.680 |
| Gender (male *vs* female) | 1.226 (0.726–2.072) | 0.446 |
| Binet stage A (*vs* C) | 0.339 (0.177–0.647) | **0.001** |
| Binet stage B (*vs* C) | 1.051 (0.548–2.013) | 0.882 |
| CD38 ≥ 30% (*vs* <30%) | 0.963 (0.535–1.731) | 0.898 |
| ZAP-70 ≥ 20% (*vs* <20%) | 2.428 (1.354–4.355) | **0.003** |
| IGHV mutation (*vs* non-mutation) | 0.787 (0.454–1.364) | 0.393 |
| p53 Mutation (*vs* non-mutation) | 0.961 (0.529–1.748) | 0.897 |
| Unfavorable karyotype† (*vs* favorable‡) | 1.337 (0.508–3.520) | 0.557 |
| Unfavorable karyotype† (*vs* others) | 0.980 (0.380–2.530) | 0.967 |
| CD20 ≥ 60.3% (*vs* <60.3%) | 0.510 (0.236–1.104) | 0.087 |

†Unfavorable karyotype, del(17p13) and del(11q22.3). ‡Favorable karyotype, del(13q14) as the sole abnormality. CI, confidence interval; IGHV, variable region of immunoglobulin genes; ZAP-70, zeta-associated protein-70. Bold values indicate statistically significant.

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## Disclosure Statement

The authors have no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Prognostic impact of CD20 on treatment-free survival in the group of patients with rituximab chemotherapy and non-rituximab treatment. Rituximab treatment affected the prognostic impact of the percentage of CD20 in patients in a naïve state of disease ($P = 0.12$; $P = 0.52$).

**Fig. S2.** Co-expression pattern of ZAP-70 and CD20 and their impact on treatment-free survival (TFS). There were significant statistical differences between groups in terms of TFS ($P = 0.01$). The low ZAP-70/high CD20 group had a longer TFS compared with other groups; the median TFS was 72 months (low ZAP-70/high CD20), 15 months (high ZAP-70/high CD20), 4 months (low ZAP-70/low CD20) and 3 months (high ZAP-70/low CD20), respectively.


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