The complex karyotype landscape in chronic lymphocytic leukemia allows to refine the risk of Richter syndrome transformation

by Andrea Visentin, Laura Bonaldi, Gian Matteo Rigolin, Francesca Romana Mauro, Annalisa Martines, Federica Frezzato, Stefano Pravato, Leila Romano Gargarella, Maria Antonella Bardi, Maurizio Cavallari, Eleonora Volta, Francesco Cavazzini, Mauro Nanni, Monica Facco, Francesco Piazza, Anna Guarini, Robin Foà, Gianpietro Semenzato, Antonio Cuneo, and Livio Trentin

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The complex karyotype landscape in chronic lymphocytic leukemia allows to refine the risk of Richter syndrome transformation

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ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the local research ethics committee of Padua hospital and informed consent was obtained from all patients.

CONFLICT OF INTEREST

AV received honoraria from Janssen, Abbvie, Italfarmaco. LT received research funding by Gilead, Roche, Janssen and Takeda, advisory board for Roche, Shire and Abbvie, Astrazeneca. GMR received research funding by Gilead. FRM advisory board for Janssen, Shire and Abbvie. AC advisory board and speaker bureau for Roche, Abbvie, Gilead and Janssen. GS board member of Abbvie, Roche, Janssen and Celgene. RF advisory board or speaker bureau for Roche, Abbvie, Celgene, Incyte, Amgen, Janssen, Gilead and Novartis.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are not publicly available due to the data protection and lack of consent from the patients. Access to data is strictly limited to the researchers who have obtained permission for data processing.

AUTHORS’ CONTRIBUTIONS

AV designed the study, performed statistical analysis, visited patients and wrote the article; SP, LRG, MC, EV and FC and provided intellectual inputs and visited patients; LB, AM, MAB and MN performed cytogenetic tests; FF, MF and AG performed cytofluorimetric and IGHV analysis; FRM, GMR, PF, GS, RF, AC and LT visited patients, provided intellectual inputs and reviewed the article.
ABSTRACT

Complex karyotype (CK) at chronic lymphocytic leukemia (CLL) diagnosis is a negative biomarker of adverse outcome. Since the impact of CK and its subtypes, namely type-2 CK (CK with major structural abnormalities) or high-CK (CK with ≥5 chromosome abnormalities), on the risk of developing Richter syndrome (RS) is unknown, we carried out a multicenter real-life retrospective study to test its prognostic impact.

Among 540 CLL patients, 107 harbored a CK at CLL diagnosis, 78 were classified as CK2 and 52 as high-CK. Twenty-eight patients developed RS during a median follow-up of 6.7 years. At the time of CLL diagnosis, CK2 and high-CK were more common and predicted the highest risk of RS transformation, together with advanced Binet stage, unmutated (U)-IGHV, 11q-, TP53 abnormalities. We integrated these variables into a hierarchical model: high-CK and/or CK2 patients showed a 10-year time to RS (TTRS) of 31%; U-IGHV/11q-/TP53 abnormalities/Binet stage B-C patients had a 10-year TTRS of 12%; while mutated (M)-IGHV without CK and TP53 disruption a 10-year TTRS of 3% (p<0.0001).

We herein demonstrated that CK landscape at CLL diagnosis allows to refine the risk of RS transformation and we recapitulated clinico-biological variables into a prognostic model.
INTRODUCTION

Chronic lymphocytic leukemia (CLL), the most common leukemia in western countries, is a remarkably heterogeneous disease, with some patients never requiring treatments and others with a highly aggressive and/or rapidly progressive clinical course (1, 2). Richter syndrome (RS) is the transformation of CLL into an aggressive lymphoma, most commonly resembling diffuse large B-cell lymphoma (DLBCL) or Hodgkin lymphoma (HL) variants (3, 4). It is characterized by fast growing lymphadenopathies, 18-fluorodeoxyglucose (FDG) positron emission tomography computerized tomography (PET-CT)-avid masses, B symptoms, worsening performance status and increased lactate dehydrogenase levels (5). It is a challenging task to distinguish RS from progressive CLL and it is even more difficult to study prognostic markers since the frequency of RS transformation affects 2-10% of CLL patients (5).

Several studies have proven that chromosome banding analysis is able to refine the prognostic stratification of CLL as compared to fluorescence in situ hybridization (FISH) analysis. In fact, 22-36% of CLL cases with “normal” FISH carry chromosomal aberration following stimulated karyotypic analyses. In particular, complex karyotype (CK), defined by the presence of at least 3 chromosome lesions in the same clone, is detectable in 14-34% of CLL cases (6-9). The presence of a CK is both a negative prognostic and predictive biomarker associated with an adverse outcome (6, 10) and worse response to chemoimmunotherapy (7, 11) as well as to novel agents (12, 13), regardless of the CLL-IPI score or IGHV mutational status (8). However, the CK itself is a heterogeneous quantitative and qualitative cytogenetic category that includes numerical (i.e. monosomies and trisomies) and structural abnormalities (i.e. balanced and unbalanced translocations, marker chromosomes, isochromosomes, deletions, insertions and additions). Recently, collaborative studies have demonstrated that among CK cases assessed at CLL diagnosis, those harboring 5 or more chromosome abnormalities (high-CK) (14) or those with major structural abnormalities, also called type-2 CK (CK2) (15, 16), identify highly aggressive disease subsets with a poor outcome; the latter is also characterized by a peculiar mRNA expression profile (15, 17). Indeed, most of the patients included in these retrospective studies were managed with chemoimmunotherapy (14-16). However, the presence of CK has been rarely associated to the development of RS (18) and to date it is unknown whether CK subtypes, namely high-CK or CK2, could help to identify patients at a higher risk of developing a RS at CLL diagnosis.

In this multicenter retrospective study, we documented for the first time that the presence of a CK at CLL diagnosis is associated with an increased risk of developing a RS.
particular, patients with CK2 and high-CK had the highest likelihood of RS transformation. Finally, we recapitulated clinico-biological variables associated with RS into a prognostic model defining 3 statistically different classes of risk of developing RS, being the lowest risk for \textit{IGHV} gene mutated (M-IGHV) patients without any CK subtypes and absence of TP53 abnormalities, and the highest risk for patients harboring highCK and/or CK2 subtypes.

**METHODS**

*Study design*

Inclusion criteria for this study were diagnosis of CLL according to the 2008 iwCLL guidelines\(^{(19)}\), histologically confirmed diagnosis of RS (diffuse large B-cell lymphoma or high-grade B-cell lymphoma), age >18 years and chromosome banding analysis performed within 1 year from diagnosis in patients without features of disease progression. Data included in the comparative analysis were gender, age, Binet stage\(^{(19)}\), CLL treatment prior to RS, 11q22-23 deletion by FISH\(^{(20)}\), \textit{IGHV} gene mutational analysis\(^{(21)}\) and \textit{TP53} abnormalities including gene deletions (deletion 17p13) or mutations\(^{(22)}\), β\(_2\)-microglobulin level >3.5mg/L. The primary endpoint was the impact of overall CK, CK2 and highCK on the time to Richter syndrome (TTRS) transformation. The correlation of RS with clinical and biologic variables and their impact on TTRS were secondary endpoints. This study was approved by the local research ethic committee and informed consents were obtained from all patients.

*Chromosome banding analysis*

Cytogenetic analysis was performed on peripheral blood after a 72h exposure to 500µM CpG ODN DSP30 (Roche, Risch, CH) mitogen + 20U/mL IL2 (Roche). Cultures were exposed overnight to 0.1 µg/mL colcemid (Gibco\® Karyomax Colcemid, ThermoFisher, Waltham, MA USA) to obtain metaphases and then harvested following standard procedures. Karyotype was described after the analysis of at least 20 G-banded metaphases using the IKAROS software (MetasYstems, Altlhusseim, Germany), according to International guidelines (ISCN 2016). The definition of a Complex karyotype (CK) was based on defined by the presence of 3 or more chromosome abnormalities in the same clone\(^{(6, 8, 23, 24)}\). According to the literature, CK2 is represented by CK cases with major structural rearrangements that are unbalanced translocations, chromosome additions, insertion, duplications, ring, dicentric and marker chromosomes, whereas, complex karyotypes with balanced translocations, deletions, monosomies or trisomies is defined as type-1 (CK1)\(^{(16)}\). High-CK cases were those
presenting at least 5 chromosome abnormalities (14). Chromosome abnormalities found in only 1 metaphase were not considered as clonal, and were excluded. Karyotype were reported by local cytogeneticist (AM, MAB and MN) and reviewed by LB and AV.

Detail description of IGHV mutational status (25-29), assessment of stereotyped B-cell receptor (BCR) (30, 31), cytogenetics by fluorescence in situ hybridization (FISH) (26, 32), TP53 gene mutation (22), and NOTCH1 c.7544_7545delCT analysis (33) are available in the supplementary methods.

**Statistical analysis**

Categorical variables were compared by the Chi-square test (for Binet stages and FISH) or the Fisher exact test (gender, treatment, TP53 and IGHV), when appropriate. Continuous variables (median age) were compared using the Mann-Whitney test. TTRS was calculated starting from the date of CLL diagnosis to RS transformation (event) or last known follow-up (censored) (19, 34). OS was calculated starting from the date of CLL or RS diagnosis, when specified, to death for any cause, or last known follow-up. Survival analyses were performed by the Kaplan-Meier method and the Log-rank test was used to compare survival curves between groups. The Cox regression model was employed to estimate hazard ratios (HR). The Cox proportional hazard assumption was assessed based on the scaled Schoenfeld residuals. The stability of our model was internally validated by the bootstrap .632 method with B=540. The Harrell concordance index (c-index; 1.0 indicates a perfect discrimination, while a value of 0.5 indicates equivalence to chance) was used to compare our prognostic model (35). The prediction error was calculated as 1 - c-index, corrected for optimism and estimated using the .632 bootstrap method (36). Akaike information criterium (AIC) was calculated using the AIC function with R (an open source statistical package downloadable from http://www.r-project.org) (37). A p value >0.05 was considered as not significant.

**RESULTS**

**Patients' characteristics.**

We gathered data from 540 treatment-naive CLL patients with chromosome banding analysis assessed within 12 months from diagnosis in three Italian centers (Table 1). The median age at diagnosis of the whole case series was 63±12 years, 61% were males, 75% showed Binet A stage, the median β2-microglobulin was 2.93mg/L, 57% of patients were U-
IGHV, 11% harbored TP53 abnormalities (8% 17p13 deletion and 3% only TP53 mutation) and 20% a CK (Figure S1A). NOTCH1 mutation was assessed in 47 patients at CLL diagnosis and it was found in 2 subjects who further developed RS. Two hundred and fifty-two patients subsequently received at least one line of therapy - 31% FCR (fludarabine, cyclophosphamide, rituximab), 17% BR (bendamustine, rituximab), 10% ibrutinib, 5% chlorambucil plus an anti-CD20 monoclonal antibody, 2% venetoclax, 35% other treatments such as FC or chlorambucil single agent as first line therapy - and 90 died during the follow-up.

According to the qualitative CK subtype, 29 out of 107 (27%) patients displayed a CK1 and 78 (73%) a CK2 (Figure S1A, Table 1). Whereas according to the number of chromosome lesions 165 (30%) patients had a normal karyotype (i.e. 46,XX or 46,XY for females and males, respectively), 268 (50%) had 1 or 2 lesions, 54 (10%) 3 or 4 abnormalities and 52 (10%) were classified as high-CK (i.e. ≥5 chromosome lesions) (Figures S1A and S2A, Table 1). In particular a high-CK was more common in CK2 than in CK1 patients, being present in 63% of patients harboring a CK2 subtype but only in 10% of CK1 patients (p<0.0001, Figure S2A).

As a preliminary step for our further analysis, we confirmed the established prognostic role of overall CK, CK with major unbalanced abnormalities (i.e. CK2) and highCK in our dataset (Figure S2B-D). The 10-year OS was 54% and 79% for CK and no-CK patients, respectively (p<0.0001, Figure S2B); 48% vs 72% vs 79% for CK2, CK1 and no-CK (p<0.0001, Figure S2C), respectively; 44% vs 64% vs 70% vs 90% for patients with ≥5 (i.e. high-CK), 4-3, 2-1 and without chromosome abnormalities (p<0.0001, Figure S2D), respectively.

Clinico-biological features of patients who developed a RS transformation.

Twenty-eight (5.2%) patients developed a histologically confirmed RS over a median follow-up of 6.7 years (Figure S1B). The median age at RS diagnosis was 68 years (range 38-84), 61% were males, 75% received a previous CLL treatment, 79% were U-IGHV, 32% presented TP53 abnormalities, 50% harbored a CK at CLL diagnosis, that included 46% and 39% of CK2 and high-CK subtypes, respectively. Eight cases showed deletion of 9p21.3, i.e. the locus of CDKN2A gene, all with a CK. In particular, 8/8 were classified as CK2 and 6/8 as high-CK subtype. Only one patient, who developed RS, received ibrutinib frontline. We also observed that more patients who developed a RS displayed an advanced Binet stage at CLL diagnosis (p=0.0113) and were enriched in U-IGHV (p=0.0191), TP53 abnormalities (p=0.0043), CK overall (p=0.0002), CK2 (p<0.0001) and high-CK (p<0.0001) cases as compared to patients who did not develop a RS (Table 1, Figure S1C). Age at CLL diagnosis (median age 63.5 and 63.3 years), gender distribution (61% both), trisomy of chromosome 12
(11% and 16%), β2-microglobulin (median levels 3.2mg/L and 2.9mg/L) and stereotyped
BCR (10.7% vs 9.8%) had a superimposable distribution among patients with and without a
RS transformation (Table 1).

**Prognosticators of Richter Syndrome**

The cumulative incidence of RS slowly increases over time. As shown in Figure 1A, the
2.6%, 12% and 13% of patients developed a RS within 5, 10 and 15 years after CLL diagnosis,
respectively. We observed that patients with a CK, overall (Figure 1B) and its subtypes
(Figure 1C-D), had a very high risk of developing a RS.

The estimated 10-year TTRS was 25% vs 8% (p<0.0001), 38% vs 8% (p<0.0001) and
41% vs 8% (p<0.0001) for patients with CK vs no-CK, CK2 vs other patients (i.e. CK1 or noCK),
highCK vs other patients (i.e. 3-4 or 1-2 or, 0 chromosome abnormalities) respectively (Figure
1C-D and S3G-H). Multivariate analysis revealed that CK overall was associated with a more
than 4-fold higher risk of developing a RS (HR 4.7, 95% CI 2.2-9.9, p<0.0001). This risk was
even higher for CK subtypes, being more than 5-fold (HR 5.6, 95% CI 2.7-11.8, p<0.0001) and
7-fold (HR 6.9, 95% CI 3.3-14.9, p<0.0001) higher for patients harboring CK2 and high-CK
subtypes, respectively (Table 2). Other variables associated with TTRS at univariate and
multivariate analysis were Binet stage B-C, U-IGHV, 11q-, TP53 abnormalities (Table 2, Figure
S3A-E).

Among CK2 and/or high-CK patients (n=81), 32 (39%) patients carried TP53
abnormalities and 52 (63%) an U-IGHV status. We found that TP53 abnormalities and IGHV
status mildly impact on the risk of developing RS among CK2 and/or high-CK subgroup, but
the difference was not statistically significant (Figure S4A-B, p=0.1150 and p=0.1405,
respectively). These data suggest that CK subtypes *per se* represent a stronger prognosticator
of RS transformation than conventional biologic markers such as TP53 disruption and U-IGHV
conformation.

The median OS from CLL diagnosis for the whole population was not reached and the
estimated 10-year OS was 73% (Figure S5A). Patients who developed a RS had a shorter OS
(Figure 2A). The median OS was 7 years vs not reached and the estimated 10-year OS was
16% vs 79% for patients who developed a RS vs those who did not transform (Figure 2E,
p<0.0001), respectively. Variables that were associated with a higher risk of death in
multivariate analysis are summarized in Table S1.

The median time from CLL diagnosis to RS transformation was 5.3 years ranging from
0.10 years to 10.8 years. Only in 1 patient RS was diagnosed within 6 months from CLL
diagnoses. The median OS from RS transformation was 5.3 months and the 2-year OS was only 20% (Figure 2B). The OS from RS was not affected by the presence of a CK at CLL diagnosis nor its subtypes (Figure S5B-C). The 2-year OS from RS was 28% vs 10% for CK2 cases and other patients (i.e. CK1 and no-CK) (p=0.3317) and 24% vs 16% for high-CK cases and other patients, respectively (i.e. <5 chromosome abnormalities) (p=0.9864) (Figure S5C-D). No traditional prognostic markers could foresee the risk of death after RS diagnosis in our population (Table S2).

A Richter syndrome prognostic model

By integrating CK subtypes, TP53 abnormalities, 11q deletion, IGHV mutational status and Binet stages based on HR values, we developed a hierarchical model leading to the identification of three statistically different groups ranked from the shortest to the longest TTRS as follows: 81 (15%) patients were classified as high-CK and/or CK2, and had a 5- and 10-year TTRS of 13% and 31%; 247 (46%) patients displayed U-IGHV status or 11q- or TP53 disruption or Binet stage B-C, and showed the 5- and 10-year TTRS of 0.9% and 12%; 212 (39%) patients were M-IGHV without CK and TP53 abnormalities, and had a 5- and 10-year TTRS of 0.7% and 3% (Figure 3, p<0.0001). Multivariate analysis confirmed that the former subgroup (i.e. high-CK and/or CK2) was associated with the highest risk of RS transformation (HR 9.2, 95% CI 3.8-46, p<0.0001) compared to the low-risk group which is characterized by the presence of M-IGHV without CK and TP53 abnormalities. Patients with U-IGHV or 11q- or TP53 abnormalities or Binet stage B-C had an intermediate risk, with a 3-fold higher risk of RS compared to low-risk patients (HR 3.4, 95% CI 1.5-7.5, p=0.0023) (Table 2). Our model was also internally validated using the bootstrap .632 method showing a prediction error of 0.26. Finally, the c-index for our proposed model was 0.81 for TTRS and the Akaike information criterium was 286. These results indicate that our model had a good prediction accuracy for the risk of developing a RS, higher than of the CLL-IPI(38) (c-index 0.69, prediction error 0.28, Akaike information criterium 301) and the Barcelona-Brno(39) (c-index 0.74, prediction error 0.25, Akaike information criterium 292) scores accuracies applied to our population (Figure S4C-D). Based on the lower Akaike score, our RS prognostic model predicts better the risk of developing a RS that the available comparators.

DISCUSSION

In this multicenter retrospective study, we demonstrated that patients harboring a CK at CLL diagnosis, in particular those with CK2 and/or high-CK, are characterized by the
highest risk of developing a RS transformation. Subsequently, by integrating data of CK subtypes with other clinical and biologic variables associated with the risk of RS we were able to define a RS prognostic model. To minimize selection and attrition biases as well as imprecise reporting of data inherent to observational studies, we asked to the clinicians to report all patients who performed stimulated cytogenetic within the first year from diagnosis. We analyzed the reported data, performed computerized and manual consistency checks on each case report form.

RS is a rare and an aggressive complication of CLL patients, affecting between 2% and 10% of CLLs(34). Most RS patients are elderly, have a poor performance status and suffer from several comorbidities which limit the use of intensive chemoimmunotherapy(40). Since the majority of patients are primary refractory to first-line treatment and only a few of them can undergo allogenic stem cell transplantation procedures, the reported estimated survival after a diagnosis of RS is usually less than 1 year even with the introduction of targeted-therapy(41, 42) and immune checkpoint inhibitors(43). For these reasons, the standard of care of patients with RS remains a primary unmet need. Known biologic risk factors for the development of RS are TP53 and CDKN2A aberrations, NOTCH1 mutation and a stereotype BCR subset #8(44, 45). So far, the impact of CK at CLL diagnosis on the risk of developing RS has been investigated in only a few studies(46, 47).

The German CLL study group has recently reviewed the clinical features of RS patients within their clinical trials(34). In this study 3.5% of CLL developed a RS transformation after a median observation time of 4.4 years. The median age at RS was 65 years and the median OS after RS was 9.4 months, which was significantly longer for HL as compared to the DLBCL variant (median OS 83 months vs 8.7 months, respectively). Adverse risk factors at trial enrollment, such as 17p13 deletion by FISH, high β2-microglobulin and CLL-IPI scores were more common in patients who developed a RS(34). While NOTCH1 mutations and stereotype #8 were not recurrent in RS cases (34). Conversely, among the 204 RS from the Mayo clinic the median OS after RS diagnosis was 12 months(48). In a multivariate Cox regression analysis, prior CLL treatment and older age, but not TP53 disruption, were associated with a shorter OS(48). The results of our real-life study are in line with the GCLLSG and Mayo clinic reports, even though our patients were slightly older, and this could explain the shorter OS in our RS cohort (median survival after RS is 5.3 months). Comparable survival rates, between 6 and 12 months, have been observed in other retrospective analyses(34, 44, 48). In addition, advanced Binet stage, U-IGHV and 11q- were also significantly associated with the risk of RS in our patients.
Chromosome banding analysis in CLL is capable of identifying chromosomal abnormalities than are missed by FISH analysis, sometimes fulfilling the criteria of CK(6, 24, 49, 50). Genomic microarrays also have emerged as a valuable tool for genome-wide studies in CLL. However, in a recent study no significant differences emerged in patients’ classification, time to first treatment, OS and prediction accuracy between chromosome banding analysis and genomic microarrays(51). The prognostic and predictive role of CK, defined by the presence of at least 3 chromosomal lesions, is evident at diagnosis(6, 8), as well as at disease progression(7) and in relapsed/refractory patients treated with ibrutinib(13, 52) or venetoclax(12). Of note, CK was not a prognostic marker of survival on multivariate analysis for patients treated frontline with ibrutinib±rituximab(53). While treatment with idelalisib plus rituximab seems to have a comparable efficacy in R/R patients with and without CK (54-56). CK has been found in 14-35% of CLL depending on the studies(6, 10), and identifies a heterogeneous cytogenetic category in terms of quantitative and qualitative characteristics. Data from the literature documented that the presence of at least 5 chromosomal aberrations is associated with a very aggressive clinical course independently of the IGHV status and TP53 lesions(14). Our collaborative group has previously demonstrated that almost 70% of CK cases harbor major structural aberrations such as unbalanced translocations, ring or marker chromosomes(15). This subset, called CK2, was associated with a higher incidence of TP53 aberrations, chemo-refractoriness, early relapse after chemoimmunotherapy, and a shorter OS at multivariate analysis(15). In addition, the prognostic and predictive accuracy of CK subtypes is enhanced when it is combined with the IGHV mutational status(16). Interestingly, a recent analysis of the international CLL14 clinical trial suggests that the fixed-duration combination of obinutuzumab plus venetoclax seems to overcome the negative predictive impact of CK, both in terms of undetectable minimal residual disease rates and progression-free survival(57).

The presence of CK has been sporadically linked to the development of RS(18). In a retrospective study on CLL patients treated with FCR, 1 out of 4 cases with RS had a CK(9). Anderson et al(12) found a CK in 48% of the 25 patients who progressed on venetoclax, including 8 of 17 patients with RS. Rogers et al(47), reported a CK in 67% patients who developed a RS and found that a CK had an adverse impact on the R-EPOCH regimen. A recent study from the Ohio State University found that 6 out of 9 patients with a near-tetraploidy (4 copies of most chromosomes) karyotype developed a RS(46). At multivariate analysis, near-tetraploidy and CK predicted ibrutinib discontinuation due to transformation(46). Although, the exact mechanism that favor the development of a RS in patients with CK is unknown, the
strong association between CK and TP53 abnormalities, short telomeres length and, consequently, the increase chromosome instability could play a relevant role\(^{(18, 58)}\).

Thanks to stimulated chromosome banding analysis we were able to identify a CK in 20\% of 540 CLL patients and could demonstrate that patients harboring a CK2 or a high-CK had a 6 and 7-fold increased risk of developing RS. We therefore suggest that the integration of CK subtypes together with the IGHV mutational status, TP53 abnormalities, 11q22-23 deletion and Binet stage can allow to refine the prognostic risk of RS transformation (Figure 3). Indeed, we could show that M-IGHV patients without any CK subtypes and a wild-type TP53 gene are characterized by a very low risk of developing RS, being only 0.7\% in 5 years from CLL diagnosis. On the other hand, patients with CK subtypes, both CK2 and/or high-CK, are characterized by the highest risk of developing RS, with 31\% of them experiencing a disease transformation within 10 years from diagnosis. In addition, our model seems to better predict the risk of RS transformation than the available scoring systems. Our results, as most data found from the literature, derived from a cohort of patients mainly treated with chemoimmunotherapy also due to their longer follow-up. Although the cumulative incidence of RS among patients treated with chemo/chemoimmunotherapy seems to be higher that patients treated with BTK or BCL2 inhibitors, this difference was not statistically significant (p=0.3337, Figure S5E). In addition, after validation by an independent cohort of patients treated frontline with targeted drugs and in a prospective study, our prognostic model might be used in the follow-up management of patients with CLL. In particular, patients with a CK2 and/or high-CK should be carefully monitored for the development of a RS during their follow-up.
REFERENCES

Visentin A, et al. Complex karyotype subtypes and RS

Table 1. Clinical and biological features of patients

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</tr>
<tr>
<td>A</td>
<td>407 (75%)</td>
<td>15 (54%)</td>
<td>392 (77%)</td>
<td>0.0113</td>
</tr>
<tr>
<td>B - C</td>
<td>133 (25%)</td>
<td>13 (46%)</td>
<td>120 (23%)</td>
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<tr>
<td><strong>β2-microglobulin (mg/L)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>median±sd</td>
<td>2.9±1.5</td>
<td>3.2±0.98</td>
<td>2.9±1.6</td>
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<td><strong>IGHV status</strong></td>
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<tr>
<td>M-IGHV</td>
<td>232 (43%)</td>
<td>6 (21%)</td>
<td>225 (44%)</td>
<td>0.0191</td>
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<tr>
<td>U-IGHV</td>
<td>309 (57%)</td>
<td>22 (79%)</td>
<td>287 (56%)</td>
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<td><strong>FISH</strong></td>
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<tr>
<td>13q or Normal</td>
<td>404 (75%)</td>
<td>19 (68%)</td>
<td>385 (75%)</td>
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<tr>
<td>+12</td>
<td>84 (15%)</td>
<td>3 (11%)</td>
<td>81 (16%)</td>
<td>0.0415*</td>
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<tr>
<td>11q -</td>
<td>52 (10%)</td>
<td>6 (21%)</td>
<td>46 (9%)</td>
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<td><strong>TP53 abn</strong></td>
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<td></td>
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<tr>
<td>Normal</td>
<td>482 (89%)</td>
<td>19 (68%)</td>
<td>463 (90%)</td>
<td>0.0043</td>
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<td>Disrupted</td>
<td>58 (11%)</td>
<td>9 (32%)</td>
<td>49 (10%)</td>
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</tr>
<tr>
<td><strong>KARYOTYPE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no CK</td>
<td>433 (80%)</td>
<td>14 (50%)</td>
<td>419 (82%)</td>
<td>0.0002</td>
</tr>
<tr>
<td>CK</td>
<td>107 (20%)</td>
<td>14 (50%)</td>
<td>93 (18%)</td>
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<tr>
<td><strong>QUALITATIVE</strong></td>
<td></td>
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<tr>
<td>no CK</td>
<td>433 (80%)</td>
<td>14 (50%)</td>
<td>419 (82%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CK1</td>
<td>29 (5%)</td>
<td>1 (4%)</td>
<td>28 (5%)</td>
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</tr>
<tr>
<td>CK2</td>
<td>78 (14%)</td>
<td>13 (46%)</td>
<td>65 (13%)</td>
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<tr>
<td><strong>QUANTITATIVE</strong></td>
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<tr>
<td>0</td>
<td>165 (30%)</td>
<td>3 (11%)</td>
<td>162 (32%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1-2</td>
<td>269 (50%)</td>
<td>11 (39%)</td>
<td>258 (50%)</td>
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<tr>
<td>3-4</td>
<td>54 (10%)</td>
<td>3 (11%)</td>
<td>52 (10%)</td>
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<tr>
<td>≥5</td>
<td>52 (10%)</td>
<td>11 (39%)</td>
<td>41 (8%)</td>
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<td><strong>RS SCORE</strong></td>
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<tr>
<td>low-risk</td>
<td>212 (39%)</td>
<td>3 (11%)</td>
<td>209 (41%)</td>
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<tr>
<td>Int.-risk</td>
<td>247 (46%)</td>
<td>12 (43%)</td>
<td>235 (46%)</td>
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<tr>
<td>high-risk</td>
<td>81 (15%)</td>
<td>13 (46%)</td>
<td>68 (13%)</td>
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RS = Richter syndrome, sd = standard deviation, M-IGHV = mutated IGHV gene, U-IGHV = unmutated IGHV gene, 11q- del11q22-23 by interphase FISH analysis, TP53 abn = TP53 abnormalities include deletions and/or mutations, CK = complex karyotype, CK1 = type-1 CK, CK2 = type-2 CK, highCK = ≥5 chromosome abnormalities, high-risk = CK2 and/or highCK, int.-risk = U-IGHV/11q-/TP53abn/Binet B-C, low-risk = M-IGHV without CK and TP53 wild type, n.a.- not applicable. * data available from 520 (96%) patients, 26 (93%) who developed a RS and 494 (96%) who did not transform. + Analysis between subgroups with 11q- and others.
Table 2. Hazard ratios (HR) for the time to Richter syndrome.

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<th>MULTIVARIATE ANALYSIS</th>
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<td>TTRS</td>
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<tr>
<td>≥65 years</td>
<td>1.4</td>
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<td>Age*</td>
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<td>Male</td>
<td>1.0</td>
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<tr>
<td>β2MG high*</td>
<td>1.8</td>
<td>0.6-5.6</td>
<td>0.2925</td>
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<tr>
<td>Binet B-C</td>
<td>3.9</td>
<td>1.6-9.6</td>
<td>0.0024</td>
</tr>
<tr>
<td>U-IGHV</td>
<td>4.0</td>
<td>1.9-8.6</td>
<td>0.0004</td>
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<td>+12</td>
<td>0.8</td>
<td>0.3-2.4</td>
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</tr>
<tr>
<td>11q-</td>
<td>4.6</td>
<td>1.3-16.7</td>
<td>0.0215</td>
</tr>
<tr>
<td>TP53 abn</td>
<td>9.5</td>
<td>2.9-31.4</td>
<td>0.0002</td>
</tr>
<tr>
<td>CK</td>
<td>7.4</td>
<td>3.0-18.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CK2</td>
<td>8.8</td>
<td>4.9-19.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>High-CK</td>
<td>9.9</td>
<td>6.5-22.9</td>
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<td>RS MODEL</td>
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<tr>
<td>Low-risk</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Int.-risk</td>
<td>4.0</td>
<td>1.4-11.4</td>
<td>0.0101</td>
</tr>
<tr>
<td>High-risk</td>
<td>13.6</td>
<td>7.1-20.9</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

TTRS = time to Richter syndrome, β2MG high = beta2-microglobulin >3.5mg/L, U-IGHV = unmutated IGHV gene, TP53 abn = TP53 abnormalities include deletions and/or mutations, CK = complex karyotype, CK1 = type-1 CK, CK2 = type-2 CK, highCK = ≥5 chromosome abnormalities, High-risk = CK2 and/or highCK, Int.-risk = U-IGHV/11q-/TP53abn/Binet B-C, Low-risk = M-IGHV without CK and TP53 wild type. n.a. = not applicable. +: age considered as continuous variable. *: data available from 520 (96%) patients, 26 (93%) who developed a RS and 494 (96%) who did not transform.
LEGEND TO FIGURES

**Figure 1. Kaplan Meyer curves of time to Richter syndrome.** The upper-left (A) panel shows the time to Richter syndrome (RS) transformation for the whole population. Patients with a CK overall (B), CK2 (C) or high-CK (D) have a risk of developing a RS significantly increased compared to the other patients (Log-rank test, p<0.0001).

**Figure 2. Kaplan Meyer curves of overall survival.** The left panel (E) shows the overall survival analysis for patients with RS transformation and those who did not develop a RS (no RS). Patients who developed a RS had a shorter survival, calculated from CLL diagnosis (Log-rank test, p<0.0001). The right (F) panel shows the overall survival after RS transformation, confirming their very poor prognosis.

**Figure 3. The Richter syndrome scoring system.** Kaplan-Meier curve of time to Richter syndrome transformation according to the Richter syndrome scoring system. Patients were classified at high-risk if they were high-CK and/or CK2 at CLL diagnosis (blue curve); at intermediate-risk if they displayed unmutated IGHV status (U-IGHV), 11q22-23 deletion (11q-), TP53 abnormalities (including deletions or mutations, TP53 abn) or Binet stage B-C (grey curve); at low-risk if they were IGHV mutated (M-IGHV) patients without CK and wild-type TP53 gene (TP53 not deleted non mutated) (orange curve).
Figure 2

A) Overall Survival

- RS
- no RS

Log-rank test, p<0.0001

B) Overall Survival

Percent of survival vs. months from RS
Figure 3

Time to Richter Syndrome

- highCK and/or CK2
- U-IGHV/11q-/TP53 abn/Binet B-C
- noCK/M-IGHV/TP53 wt

Log-rank test, p<0.0001

<table>
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<th></th>
<th>-</th>
<th>0.0006</th>
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<td>highCK and/or CK2</td>
<td>-</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td>U-IGHV/11q-/TP53 abn/Binet B-C</td>
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<td>-</td>
<td>0.0101</td>
</tr>
<tr>
<td>noCK/M-IGHV/TP53 wt</td>
<td>&lt;0.0001</td>
<td>0.0101</td>
<td>-</td>
</tr>
</tbody>
</table>

years from the diagnosis
The complex karyotype landscape in chronic lymphocytic leukemia allows to refine the risk of Richter syndrome transformation

Andrea Visentin¹,², Laura Bonaldi³, Gian Matteo Rigolin⁴, Francesca Romana Mauro⁵, Annalisa Martines³, Federica Frezzato¹,², Stefano Pravato¹,², Romano Gargarella Leila¹,², Maria Antonella Bardi⁴, Maurizio Cavallari⁴, Eleonora Volta⁴, Francesco Cavazzini⁴, Mauro Nanni⁵, Monica Facco¹,², Francesco Piazza¹,², Anna Guarini⁵, Robin Foà⁵, Gianpietro Semenzato¹,², Antonio Cuneo⁴, Livio Trentin¹,²,⁺.

¹Hematology and Clinical Immunology Unit, Department of Medicine, University of Padua, Padua, Italy. ²Veneto Institute of Molecular Medicine, Padua, Italy. ³Immunology and Molecular Oncology Unit, Veneto Institute of Oncology IOV-IRCSS, Padua, Italy. ⁴Hematology section, Department of Medical Sciences, Azienda Ospedaliera-Universitaria, Arcispedale S. Anna, University of Ferrara, Ferrara. ⁵Hematology division, Department of Translational and Precision Medicine, “Sapienza” University, Rome, Italy.

SUPPLEMENTARY FILES

- Supplementary methods pag. 2
- Legends to figures pag. 3
- Supplementary table S1 pag. 4
- Supplementary table S2 pag. 4
SUPPLEMENTARY METHODS

IGHV mutational status
Analysis of the IGHV mutational status was performed within 12 months from diagnosis on peripheral blood CLL cells from fresh samples or frozen purified CLL cells harvested in DMSO. RNA was extracted from 2x10^6 B cells using the RNeasy™ Total RNA kit (Qiagen, Hilgen, Germany) and reverse transcribed using the SuperScript™ Preamplification System for first-strand cDNA synthesis (Life Technologies, Carlsbad, CA). The CLL B-cell HV gene family was assigned as previously described(25, 26). HV gene sequences were determined by amplifying 5μl of the original cDNA using the appropriate HV leader and HC primers. PCR products were directly sequenced after purification with the Wizard PCR Preps (Promega, Madison, WI) using an automated genetic analyzer (3130 ABI Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using the IMGT/VQUEST and BLAST softwares(27) to detect VDJ junction. Cases with a sequence homology <98 from the corresponding germline gene were considered as mutated (M-IGHV), and those with a homology ≥98% as unmutated (U-IGHV)(28, 29). Stereotyped B-cell receptor (BCR) was assessed with ARResT(30, 31).

Cytogenetics by fluorescence in situ hybridization (FISH) and mutations
FISH was performed on standard cytogenetic preparations from peripheral blood(26). The slides were hybridized with the multicolor probe set LSI p53/LSI ATM and LSI D13S319/LSI 13q34/ CEP12 and RP11-17708 according to the manufacturer’s instructions(32). Three hundred interphase nuclei were analyzed for each probe and the cut-off for positive value was 10% for deletion of 11q22.3 (ATM), 17p13.1 (TP53) loci and 13q14.3 (D13S319), and 5% for trisomy 12. TP53 gene sequencing was performed according to ERIC guideline assessing exons 4-10; if negative exons 2, 3 and 11 were also investigated(22). NOTCH1 c.7544_7545delCT mutation was performed according to Rossi D et al (33) following local policy.
**LEGENDS TO FIGURES**

**Figure S1. Prevalence of CK subtypes and RS.** In the upper panel (A) there are apple-pie graphics of the complex karyotype (CK) rate in the whole population, on the left, and CK qualitative and quantitative subtypes on the right. The percentage of CK subtypes refers to 20% of all patients. In the middle panel (B), there is an apple pie-graphic showing the prevalence of Richter syndrome (RS) in our study population. In the bottom panel, there is a bar graph comparing clinico-biological features of patients who developed RS and those who did not transform (no RS). Fisher exact-T test, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001.

**Figure S2. Bar graph and Kaplan-Meier curves according the presence of complex karyotype and its subsets.** In the upper part of the figure we report a bar graph (A) showing the prevalence of chromosome abnormalities in the whole population and according to the qualitative classification of complex karyotype [i.e. no complex karyotype (no-CK), type-1 CK (CK1), type-2 CK (CK2)]. The presence of at least 5 chromosome abnormalities (high-CK) is rare in the whole CLL population, it is common in patients with CK2 (p<0.0001). In the middle and bottom there are Kaplan-Meier curves for overall survival from CLL diagnosis according to the IGHV mutational status (B, mutated vs unmutated IGHV genes), qualitative CK subtypes (C, type 2 CK vs type 1 CK vs no CK), quantitative CK subtypes (D, 0 vs 1-2 vs 3-4 vs ≥5 chromosome abnormalities).

**Figure S3. Kaplan-Meier curves of time to Richter syndrome according to clinico-biological variables.** We reported time to Richter syndrome according to gender (A, male vs female), age (B, ≥65 years vs <65 years), Binet stage (C, stage B-C vs stage A), deletion 11q22-23 (11q-) by FISH (D, 11q- vs +12 vs other FISH results (i.e. 13q- and normal FISH)), IGHV mutational status [E, M-IGHV (mutated IGHV gene) vs U-IGHV (unmutated IGHV gene)], TP53 abnormalities (TP53 abn, including deletions and mutations) [F, TP53 abn vs TP53 wild-type], qualitative CK subtypes (G, CK2 vs CK1 vs no CK) and quantitative CK subtypes (0 vs 1-2 vs 3-4 vs ≥5 chromosome abnormalities).

**Figure S4. Kaplan-Meier curves for time to Richter syndrome of CK2 and/or high-CK patients according to the presence of TP53 abnormalities or IGHV mutational status, and time to Richter syndrome according to different prognostic models.** In upper panels we report time to Richter syndrome transformation of CK2 and/or high-CK patients according to the presence of TP53 abnormalities (TP53 abn, including deletions and mutations) or not (TP53 wild type) (A), and according to the IGHV mutational status (M-IGHV, mutated, or U-IGHV, unmutated) (B). The increased risk of developing Richter syndrome of CK2 and/or high-CK patients is independent from TP53 abnormalities or U-IGHV status. In the lower panels there are Kaplan-Meier curves according to the CLL-IPI, on the bottom-left (C), and Barcelona-Brno scores, on the bottom-right (D) applied to the whole CLL study population.

**Figure S5. Kaplan-Meier curves for overall survival analysis.** In the upper-left panel (A) is reported the overall survival from CLL diagnosis for the whole population. Panels B and C show overall survival from RS diagnosis according to the presence of CK2 (type-2 complex karyotype), high-CK (≥5 chromosome abnormalities). In the bottom-left panel (D) there is overall survival analysis from RS diagnosis according to our proposed RS scoring system; high-risk defines patients with CK2 and/or high-CK; intermediate risk were patients with U-IGHV/TP53 abnormalities/11q-/Binet B-C; at low-risk were patients with M-IGHV without CK and TP53 wild-type. The very poor prognosis of patients with RS is not influenced by CK2 nor high-CK nor our scoring system predicts survival after RS transformation. In the bottom-right panel (E) we reported the time to Richter
syndrome for patients who were untreated during the follow-up, those treated with chemo-
chemoimmunotherapy (CT or CIT), a those treated with BTK or BCL2 inhibitors.

**TABLES**

**Table S1. Univariate and multivariate analysis for overall survival from CLL diagnosis**

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<th>MULTIVARIATE ANALYSIS</th>
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<td>HR</td>
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<td><strong>MALE</strong></td>
<td>1.48</td>
<td>0.97-2.26</td>
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<td><strong>AGE&gt;65yy</strong></td>
<td>4.87</td>
<td>3.11-7.63</td>
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<td>4.06</td>
</tr>
<tr>
<td><strong>Binet B-C</strong></td>
<td>2.31</td>
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<td>0.0009</td>
<td>2.06</td>
</tr>
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<td><strong>B2MG high</strong></td>
<td>1.83</td>
<td>1.23-2.97</td>
<td>0.0142</td>
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<td><strong>U-IGHV</strong></td>
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<td>&lt;0.0001</td>
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* B2MG high = beta2-microglobulin >3.5mg/L, U-IGHV = unmutated IGHV gene, 11q- = deletion of 11q22-23 by FISH, TP53 abn = TP53 abnormalities include deletions and/or mutations, CK = complex karyotype, CK2 = type-2 CK, highCK = ≥5 chromosome abnormalities, RS = Richter syndrome. * data available from 520 (96%) patients, 26 (93%) who developed a RS and 494 (96%) who did not transform.

**Table S2. Univariate and multivariate analysis for overall survival from RS**

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<td>p values</td>
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</tbody>
</table>

* B2MG high = beta2-microglobulin >3.5mg/L, U-IGHV = unmutated IGHV gene, PRE. THER. = previous CLL treatment, 11q- = deletion of 11q22-23 by FISH, TP53 abn = TP53 abnormalities include deletions and/or mutations, CK = complex karyotype, CK2 = type-2 CK, highCK = ≥5 chromosome abnormalities, RS = Richter syndrome. * data available from 520 (96%) patients, 26 (93%) who developed a RS and 494 (96%) who did not transform.
Figure S1

A

Distribution of CK

- CK: 20%
- no CK: 80%

Qualitative CK subtypes

- CK1: 27%
- CK2: 73%

Quantitative CK subtypes

- 3: 31%
- 4: 49%
- ≥5: 21%

B

Cases of Richter syndrome

- RS: 5%
- no RS: 95%

C

Clinico-biological features

- Male
- Binet B-C
- U-IGHV
- TP53 abn
- CK
- CK2
- highCK

- RS: *
- no RS: **
- RS: ***
- no RS: ****
- RS: ****
- no RS: ****
Figure S2

A

Number of Chromosome Abnormalities

****

B

Overall Survival

Log-rank test, p<0.0001

C

Overall Survival

Log-rank test, p<0.0001

D

Overall Survival

Log-rank test, p<0.0001
Figure S4

**A** Time to Richter Syndrome
CK2 and/or highCK patients

- TP53 dis
- TP53 wt

Log-rank test, p=0.1405

**B** Time to Richter Syndrome
CK2 and/or highCK patients

- U-IGHV
- M-IGHV

Log-rank test, p=0.1405

**C** CLL-IPI score
Time to Richter Syndrome

- > 7
- 4 - 6
- 2 - 3
- 0 - 1

Log-rank test, p<0.0001

**D** Barcellona-Brno score
Time to Richter Syndrome

- U-IGHV and 11q- / 17p-
- U-IGHV or 11q- / 17p-
- M-IGHV without 11q- / 17p-

Log-rank test, p<0.0001
Figure S5

A. Overall Survival

B. Overall Survival

C. Overall Survival

D. Overall Survival

E. Time to Richter Syndrome