The combination of complex karyotype subtypes and IGHV mutational status identifies new prognostic and predictive groups in chronic lymphocytic leukaemia

Andrea Visentin, Laura Bonaldi, Gian Matteo Rigolin, Francesca Romana Mauro, Annalisa Martines, Federica Frezzato, Silvia Imbergamo, Edoardo Scomazzon, Stefano Pravato, Maria Antonella Bardi, Maurizio Cavallari, Eleonora Volta, Francesco Cavazzini, Maurizio Nanni, Ilaria Del Giudice, Monica Facco, Anna Guarini, Gianpietro Semenzato, Robin Foà, Antonio Cuneo and Livio Trentin

BACKGROUND: Complex karyotype (CK) is a heterogeneous category with a negative impact in chronic lymphocytic leukaemia (CLL). Our group has recently reported that CK patients with major structural abnormalities (i.e. CK2) are characterised by a worse prognosis, as compared to other lesions within CK(CK1).

METHODS: We performed a multicentre retrospective study to test whether the combination of CK subtypes with IGHV status could be a relevant prognostic and predictive tool.

RESULTS: Among 522 patients 13% harboured CK2, 41% CK1 and/or U-IGHV (U-CK1) and 46% M-IGHV without any CK subtypes (M-noCK). After a median follow-up of 5.8 years, CK2 patients had the shortest TTFT (5-year TTFT 31%, 39 and 81%, p < 0.0001) and OS (5-year OS 67%, 85 and 93%, p < 0.0001) as compared to U-CK1 or M-noCK cases, regardless of TP53 abnormalities. CK2 patients also had the worst outcome after chemoimmunotherapy. In fact, the median TTNT after FCR or BR was 1.86 and 4.79 years for CK2 and U-CK1, but not reached for M-noCK patients (p < 0.0005).

CONCLUSIONS: We herein suggest that the combined assessment of the IGHV mutational status and CK subtypes refines the prognostication of CLL, allowing to identify M-IGHV patients without any CK subtypes who are characterised by an indolent disease and excellent outcome after chemoimmunotherapy.

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METHODS

Study design

Inclusion criteria for this study were diagnosis of CLL according to the 2008 iwCLL criteria, age >18 years and chromosome banding analysis performed within 1 year from diagnosis. Data included in the comparative analysis were gender, age, Binet stage, need for chemotherapy, CD38 expression (performed as previously reported with a cut-off value of 30%), cytogentic analysis detected by fluorescence in situ hybridisation (FISH), IGHV mutational analysis and TP53 abnormalities including gene deletions or mutations. The primary endpoint was the impact of the combination of CK subtypes with IGHV status on the overall survival (OS) of patients. The correlation with clinico-biological variables and its impact on time to first treatment (TTF) and relapse after chemoimmunotherapy were considered as secondary endpoints. This study was approved by the local research ethics committee and informed consent was obtained from all patients.

Chromosome banding analysis

Cytogenetic analysis was performed on peripheral blood after a 72 h exposure of 500 µM CpdG ODN DSP30 (Roche, Risch, CH) and mitogen + 20 U/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 they were harvested following standard procedures. Karyotype was described after the analysis of at least 25 G-banded metaphases using the IKAROS software (MetasYstems, Altlhussem, Germany), according to International guidelines (ISCN 2016). Complex karyotype (CK) was defined by the presence of three or more chromosome abnormalities in the same clone. Moreover, based on the type of chromosome changes among CK, we termed Type-2 CK (CK2) those cases with major structural rearrangements (i.e. unbalanced translocations, chromosomes addition, insertion, duplications, ring, dicentric and marker chromosomes). Whereas, complex karyotypes with balanced translocations, deletions, monosomies or trisomies were called as type 1 (CK1).

IGHV mutational status

Analysis of the IGHW 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 2 × 10⁶ B cells using the RNaseasy™ 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. Additionally, IGHW gene sequences were determined by amplifying 5 µl of the original cDNA using the appropriate HV leader and HC primers. PCR products were sequenced directly after purification with Wizard PCR Preps (Promega, Madison, WI) using an automated genetic analyser (3130 ABI Applied Biosystems, Foster City, CA, USA). Sequences were analysed using the IMGT/VQUEST and BLAST software to detect VDJ junction. Sequences homology <98% from the corresponding germline gene, were considered mutated (M-IGHV), as opposite to unmutated (U-IGHV) cases.

Cytogenetic by fluorescence in situ hybridisation (FISH) and TP53 mutations

FISH was performed on standard cytogenetic preparations from peripheral blood. The slides were hybridised with the multi-colour probe sets LSI p53/LSI ATM and LSI D13S319/LSI 13q34/CEP12 (Vysis-Abbott, Des Plaines, IL, USA), according to the manufacturer’s protocol. Three hundred interphase nuclei were analysed for each probe and the cut-off for positive values were 10% for deletion of 11q22.3 (ATM), 13q14.3 (D13S319) and 17p13.1 (TP53) loci and 5% for trisomy 12. High-risk FISH refers to 11q- or 17p-. As opposite, Low-risk FISH included 13q14 deletion or normal FISH. Patients harbouring trisomy 12 were considered at intermediate risk. TP53 gene sequencing between and analysis were performed according to ERIC guidelines.

RESULTS

Patients’ characteristics

We gathered data from 522 CLL patients with chromosome banding analysis and IGHW status assessed within 12 months from diagnosis (Table 1). The median age at diagnosis of the whole population was 65 years, 61% were males, 76% at Binet A stage, the median β2-microglobulin was 3.27 mg/L, 47% U-IGHV, 9% patients harboured TP53 abnormalities and 19% a CK. Two hundred and thirty-two patients received at least one line of therapy (31% FCR, 16% BR, 8% ibrutinib, 5% chlorambucil-antiCD20, 40% other treatments such as FC or chlorambucil alone, etc.) and 80 died over a median follow-up of 5.8 years. According to the subtype of CK, 30 (30%) showed a CK1 and 69 (79%) a CK2. In this latter group 35% were M-IGHV and 65% were non-mutated conformation of IGHW gene (Table 1).

As a preliminary step for our further analysis, we confirmed the established prognostic role of U-IGHV, CK and CK with major unbalanced abnormalities (i.e. CK2) in our dataset (Fig. S1A-F). The 10-year OS was 60% in U-IGHV and 89% M-IGHV group (p < 0.0001, Fig. S1D); 58% for CK and 79% for no-CK patients, respectively (p < 0.0001, Fig. S1E); 49% vs 66% vs 79% for CK2, CK1 and no-CK (p < 0.0001, Fig. S1F), respectively. Due to the superimposable
trend and absence of any statistical difference between OS curves of the CK1 and U-IGHV groups, these patients were grouped and analysed together (U-CK1), as well as M-IGHV and no-CK patients (M-noCK) (Figure S1G). Sixty-nine (13%) patients of the whole population harboured CK2, 213 (41%) CK1 or U-IGHV (U-CK1) and 240 (46%) M-IGHV without any subtype of CK (M-noCK). The former group was characterised by a more advanced stage at diagnosis (Binet C, 13% vs 7% vs 4%, \( p < 0.0001 \)), higher levels of \( \beta \)-2-microglobulin (2.47 mg/L vs 3.17 mg/L vs 3.27 mg/L, \( p < 0.0001 \)), lower number of cases with low-risk FISH (i.e. 13q- or normal FISH, 38% vs 55% vs 92%, \( p < 0.0001 \)), but an increased prevalence of \( TP53 \) aberrations (38% vs 8% vs 3%, \( p < 0.0001 \)) and number of chromosomal abnormalities (≥5 lesions, 0% vs 1% vs 59%), as compared to the other two groups (Table 1).

### Table 1. Clinical and biological features of patients

|                      | Population (n = 522) | M-noCK (n = 240) | U-CK1 (n = 213) | CK2 (n = 69) | \( p \) values |
|----------------------|----------------------|------------------|----------------|-------------|--------------|
| Gender               |                      |                  |                |             |              |
| Female               | 203 (39%)            | 88 (37%)         | 87 (41%)       | 28 (41%)    | 0.6298       |
| Male                 | 319 (61%)            | 152 (63%)        | 126 (59%)      | 41 (59%)    |              |
| Age (years)          | Median ± sd          | 65 ± 10          | 59 ± 11        | 65 ± 12     | 70 ± 10      | 0.0053       |
| Binet stage          |                      |                  |                |             |              |
| A                    | 396 (76%)            | 164 (69%)        | 189 (89%)      | 43 (62%)    | \(< 0.0001\) |
| B                    | 91 (17%)             | 66 (27%)         | 8 (4%)         | 17 (25%)    |              |
| C                    | 35 (7%)              | 10 (4%)          | 16 (7%)        | 9 (13%)     |              |
| \( \beta \)-2-microglobulin (mg/L) | Median ± sd          | 2.92 ± 1.53       | 2.47 ± 1.55       | 3.17 ± 1.34       | 3.27 ± 1.78       | \(< 0.0001\) |
| CD38a                | \(<30\%\)            | 388 (74%)        | 207 (88%)      | 140 (67%)   | 41 (62%)     | \(< 0.0001\) |
| \( \geq 30\%\)       | 121 (23%)            | 27 (12%)         | 69 (33%)       | 25 (38%)    |              |
| IGHV status          |                      |                  |                |             |              |
| M-IGHV               | 279 (53%)            | 240 (100%)       | 15 (7%)        | 24 (35%)    | n.a.         |
| U-IGHV               | 243 (47%)            | 0 (0%)           | 198 (93%)      | 45 (65%)    |              |
| FISHa                |                      |                  |                |             |              |
| Normal               | 153 (29%)            | 86 (38%)         | 59 (28%)       | 8 (12%)     | \(< 0.0001\) |
| 13q+12               | 196 (38%)            | 122 (54%)        | 56 (27%)       | 18 (26%)    |              |
| +11                  | 76 (15%)             | 17 (8%)          | 52 (25%)       | 7 (10%)     |              |
| 11q+17p              | 50 (10%)             | 4 (2%)           | 33 (16%)       | 13 (19%)    |              |
| 17p+                  | 36 (7%)              | 3 (1%)           | 10 (5%)        | 23 (33%)    |              |
| TP53a                |                      |                  |                |             |              |
| Normal               | 469 (90%)            | 229 (97%)        | 197 (92%)      | 43 (62%)    | \(< 0.0001\) |
| Abnormal             | 48 (9%)              | 6 (3%)           | 16 (8%)        | 26 (38%)    |              |
| N. Chr. abn.         |                      |                  |                |             |              |
| 0                    | 167 (32%)            | 117 (49%)        | 50 (24%)       | 0 (0%)      | \(< 0.0001\) |
| 1-2                  | 258 (49%)            | 123 (51%)        | 135 (63%)      | 0 (0%)      |              |
| 3-4                  | 53 (10%)             | 0 (0%)           | 25 (12%)       | 28 (41%)    |              |
| ≥5                   | 44 (9%)              | 0 (0%)           | 3 (1%)         | 41 (59%)    |              |

sd standard deviation, M-IGHV mutated IGHV gene, U-IGHV unmutated IGHV gene, N. Chr. abn. number of chromosomal abnormalities, CK complex karyotype, M-noCK M-IGHV without CK, U-CK1 U-IGHV and/or type 1 CK, CK2 type 2 CK. n.a. not applicable

*Missing data = 3% about CD38 expression, 1% cytogenetic by FISH and 1% for TP53 abnormalities (including deletions and/or mutations)

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and 0.63 (Fig. S3C-D), CLL-IPI 0.67 and 0.62 (Fig. S3E-F) while for our proposed model they were 0.70 and 0.69 for TTFT and OS, respectively. These results indicate that our model was slightly better than other commonly used prognostic scores applied to our population.

Predictive impact of CK subtypes and IGHV combination

The combination of complex karyotype subtypes and IGHV mutational status also provides predictive information after first-line therapy \((n = 160\) patients, \(p < 0.0001\) for both TTFT and OS, Fig. S2G-H). In particular, focusing on 107 patients treated with FCR or BR, 20\% were M-noCK, 67\% U-CK1 and 13\% CK2. We observed that only one of the M-noCK cases relapsed and that no patient has died after a median follow-up of 43 months as compared with the other two subgroups (Fig. 1e, f). The median TTNT was 1.86 and 4.79 years for CK2 and U-CK1, but not reached for M-noCK patients \((p < 0.0005, \text{Fig. 1e})\). The estimated 3-year TTNT was 92\%, 69 and 23\% for M-noCK, U-CK1 and CK2 patients \((p = 0.0005, \text{Fig. 1e})\), respectively. The median OS was 3.5 years for CK2 but not reached for both U-CK1 and M-noCK cases \((p = 0.0006, \text{Fig. 1f})\). The 3-year OS was 100\%, 94 and 62\% for M-noCK, U-CK1 and CK2 patients \((p = 0.0006, \text{Fig. 1f})\), respectively. Variables associated with TTNT and OS at univariate and
multivariate analysis were reported in Table S3 and S4. CK2 predicted a shorter TTNT and OS also at multivariate analysis (p = 0.0055 and p = 0.0113, respectively).

Subsequently, we compared CK2 patients treated with FCR or BR (n = 14) with those who received ibrutinib first-line (n = 7). Although the 3-year TTFT and OS for patients treated with chemoimmunotherapy and those with ibrutinib were 67% vs 22% and 100% vs 62%, respectively, these differences were not statistically significant (p = 0.2479 for TTNT and p = 0.2011 for OS, Fig. S4A-B).

DISCUSSION

Chromosome banding analysis in CLL is capable of identifying chromosomal abnormalities that are missed by FISH analysis, sometimes fulfilling the criteria of CK. In this retrospective study we confirmed in a large cohort of patients that CK is not a single entity but is a quantitative and qualitative cytogenetic heterogeneous category. CK patients with major structural lesions (i.e. CK2) have a dismal outcome. Furthermore, the combination of IGHV mutational status with data derived from chromosome banding analysis allows to identify a subset of patients characterised by M-IGHV without any CK subtypes with a very indolent disease, 90% alive after 10 years of follow-up, who can achieve long-term remission after a short-course of chemoimmunotherapy.

The availability of BCR- and BCL2-inhibitors, alone or in combination, is able to overcome some of the poor-risk prognostic factors associated with CLL, such as clinical stage, TP53 abnormalities and U-IGHV, and new predictive parameters are now emerging. In recent years, the prognostic and predictive role of CK, defined by the presence of at least three chromosomal lesions, is becoming evident at diagnosis, and in relapsed/refractory patients treated with ibrutinib or venetoclax. Although CK is found in 14–35% of CLL depending on the studies, it is a heterogeneous cytogenetic category from a quantitative and qualitative point of view. Data from the literature have documented that patients with +12, −18 and +19 although resembling a CK are characterised by an indolent CLL with peculiar clinical features (i.e. female predominance, young age at diagnosis, etc.). On the other hand the presence of at least five chromosomal aberrations predicted for a very aggressive clinical course independently of the IGHV status and TP53 lesions. The number of chromosomal lesions was also assessed in our study population, confirming that patients 5 or more aberrations had the shortest TTFT and OS (Fig. S5). Recently, our collaborative group has demonstrated that almost 70% of CK cases harboured major structural abnormalities (such as unbalanced translocations, ring or marker chromosomes). This subset, herein called CK2, was associated with a higher incidence of TP53 aberrations, chemo-refractoriness and a shorter OS at multivariate analysis. Furthermore, CK2 CLL cases have a distinct mRNA expression profile with a deregulation of genes involved in cell-cycle control and DNA damage response. In the present study we included 522 patients and we combined data derived from stimulated chromosome banding analysis with the IGHV mutational status in order to improve the prognostic and predictive power of these markers. We demonstrated that M-IGHV patients without any CK subtypes at diagnosis, corresponding to 45% of our cohort, are characterised by a very indolent disease with a median TTFT of 19 years and more 90% of them still alive after 10 years from diagnosis. Although patients with CK2 was a relatively rare subgroup, representing 13% of the cases, most of them (81%) required a treatment within 5 years from the diagnosis, almost all needed a second line of treatment after 3 years from chemoimmunotherapy and the median OS was 7 years.

Although the exact mechanisms which favour the development of a CK are unknown, the strong association between CK and TP53 disruption herein and by other authors, could play a relevant role. Patients with TP53 mutations are characterised by short telomeres and high hTERT expression, a condition known to cause chromosome instability. Patients with TP53 disrupted showed telomere deletion and chromosomal end-to-end fusion in cells with CK. Thomay et al. reported that loss or mutation of TP53 caused an increased number of chromosomal break events leading to dicentric chromosome and whole-arm translocation. In addition, a recent paper from the German CLL Study Group found an association between short telomere length, TP53 abnormalities, early relapse after chemoimmunotherapy and adverse survival. In particular, cases with 17p- or TP53 mutations had the shortest telomeres length, increase genomic complexity as well as clonal evolution.

The challenge of contemporary CLL treatment involves attempts to tailor therapy according to the patients’ specific biological risk profile. To responsibly and effectively advance the development of new targeted therapies, novel drugs should be specifically offered to patient subgroups who can gain the greatest benefit compared with established chemoimmunotherapy strategies. Rossi et al. demonstrated that OS of M-IGHV patients without 11q or 17p deletions is superimposable to that of the age-matched general population, while U-IGHV subjects and those with high-risk FISH aberrations (i.e. 11q- or 17p–) invariable relapsed after FCR. This observation has been also confirmed in the re-analysis of pivotal clinical trials from the German CLL study group and MD Cancer institute. Gentile et al. published a multicentre retrospective study on BR in treatment-naïve patients showing that, also with this chemoimmunotherapy, M-IGHV CLL without 11q/17p deletions experienced the best disease control and OS. All these observations clearly identified CLL patients with a low-risk biological profile who can achieve excellent long-term results and disease control with six cycles of chemoimmunotherapy. Our data confirm the above results and extend the observation supporting the notion that front-line chemoimmunotherapy, FCR or BR, represents a highly effective treatment option for physically fit M-IGHV CLL patients without a CK.

Given the disappointing results of CK2 patients with chemoimmunotherapy, we hypothesised that new agents, ibrutinib, would improve the outcome of this unfavourable subset. We compared CK2 patients treated with FCR or BR and those who received ibrutinib as first-line therapy. Although we found some differences between TTNT and OS curves, these were not statistically significant, likely related to the short follow-up (14 months for ibrutinib-treated patients) and the low number of patients (seven

| Table 2. Hazard ratios (HR) for the combination of IGHV mutational status with CK subtypes |
|------------------------------------------|----------|----------|----------|----------|----------|----------|
|                                | Univariate analysis |                   | Multivariate analysis |                   |
|                                | HR      | 95% C.I. | p values | HR      | 95% C.I. | p values |
|--------------------------------|---------|----------|----------|---------|----------|----------|
| TTFT                           |          |          |          |         |          |          |
| M-noCK                         | 1.00    |          |          | 1.00    |          |          |
| U-CK1                          | 4.31    | 3.14–5.90| <0.0001  | 3.98    | 2.87–5.52| <0.0001  |
| CK2                            | 4.89    | 2.99–7.99| <0.0001  | 5.12    | 3.5–7.47 | <0.0001  |
| OS                             |          |          |          |         |          |          |
| M-noCK                         | 1.00    |          |          | 1.00    |          |          |
| U-CK1                          | 3.10    | 1.81–5.30| <0.0001  | 3.14    | 1.75–5.64| 0.0001   |
| CK2                            | 7.07    | 3.13–15.08 | <0.0001  | 7.37    | 3.97–13.69| <0.0001  |

95% CI, 95% confidential interval; M-noCK mutated IGHV without complex karyotype, U-CK1 unmutated IGHV and/or type 1 complex karyotype, CK2 type 2 complex karyotype, TTFT time to first treatment, OS overall survival
CK2 cases treated with ibritinib). The best first and subsequent therapies for patients with CK are still matter of debate. While the presence of CK has been associated to early relapse in relapsed/ refractory patients treated with ibritinib or venetoclax,\textsuperscript{10,11} the activity of ibritinib in treatment-naive subjects with a CK has so far not been reported. Overall the literature and current data support the importance of evaluating IGHV mutational status accordingly to recently updated ivCLL guidelines, and suggest that the outcome of CK patients with chemoinmunotherapy is disappointing due to a high rate of chemo-refractoriness, early relapse and short survival.\textsuperscript{5,9}

CONCLUSIONS
Thanks to stimulated cytogenetic analysis we identified a CK in 19\% of 522 CLL patients. We herein suggest that the combination of IGHV mutation and data derived from chromosome banding analysis allows to refine the prognostic stratification of CLL, to identify M-IGHV patients without any CK subtypes who are characterised by an indolent disease and excellent outcome after chemoinmunotherapy. At the other end, CK2 patients are enriched in cases with TP53 abnormalities, have unsatisfactory responses to chemotherapy and aggressive diseases with only 40\% alive after 10 years of follow-up. New clinical trials incorporating a combination, or a sequence of novel agents should be envisaged for patients with CK, in particular with the CK2 subtype.

AUTHOR CONTRIBUTIONS
A.V. designed the study, performed statistical analysis, visited patients and wrote the article; S.i., E.S., S.F., M.C., E.V. and F.C. and provided intellectual inputs and visited patients; L.B., A.M., M.A.B. and M.N. performed cytogenetic tests; F.F., I.D.G., F.M. and A.G. performed cyt fluorimetric and IGHV analysis; F.R.M., G.M.R., G.S., R.F., A.C. and L.T. visited patients, provided intellectual inputs and reviewed the article.

ADDITIONAL INFORMATION
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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.

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Consent to publish: All patients gave consent to the publication of anonymous data.

Data availability: The datasets generated and analysed 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.

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