Mutational status of *IGHV* is the most reliable prognostic marker in trisomy 12 chronic lymphocytic leukemia

Trisomy 12 is a recurrent cytogenetic abnormality that occurs in 15-20% of Chronic Lymphocytic Leukemia (CLL).[1,2] Within the hierarchical model proposed by Döhner *et al.*,[3] trisomy 12 CLL (tris12 CLL) carry an intermediate prognostic risk, with median overall survival (OS) and time to first treatment (TTFT) usually shorter than those cases bearing the 13q14 deletion (del13q) or the so-called normal karyotype cases (i.e., lacking tris12, del13q, 11q22 deletion -del11q- and 17p13 deletion -del17p-), but longer than CLL characterized by del11q and del17p.[3] Other reports suggest a certain degree of clinical heterogeneity, with a higher incidence of second malignant neoplasms[4] and Richter transformation.[5] Tris12 CLL often show atypical morphology and immunophenotype,[4,6] frequently express CD49d[7,8] and CD38,[4,8] and bear *NOTCH1* mutations[8] and unmutated (UM) *IGHV* genes,[4] although the real impact of these CLL negative prognosticators in the tris12 setting remain to be fully elucidated. To address this issue we evaluated the prognostic impact of *IGHV, TP53, NOTCH1, SF3B1* and *BIRC3* gene mutations, along with the expression CD49d, CD38 and Zeta-chain-associated protein kinase 70 (ZAP-70) in a large tris12 CLL series and a comparably wide control series of CLL bearing del13q or a normal karyotype.

The study, approved by the Internal Review Board of the Aviano Centro di Riferimento Oncologico (Approval n. IRB-05-2010 and n. IRB-05-2015), included a multicenter series of 398 tris12-bearing CLL (*Online Supplementary Table S1*). The control series was composed of 553 CLL cases bearing either the del13q only (308 cases) or lacking tris12, del13q, del11q, del17p (245 cases). All CLL samples were from treatment-naive patients, collected at diagnosis or before progression. CLL was diagnosed according to the current International Workshop on CLL (iwCLL) guidelines.[9] Patient characterization included modified Rai stage, expression of CD49d (CD49d positive: ≥30%), CD38 (CD38 positive: ≥30%) and ZAP-70 (ZAP-70 positive: ≥20%), *IGHV* mutational status (mutated if ≥2% mutations from germ line sequence), and *TP53, BIRC3, NOTCH1, SF3B1* mutations (mutated if >1%). Interphase fluorescent *in situ* hybridization (FISH) on peripheral blood investigated tris12, del13q, del11q, del17p; cases bearing each aberration in ≥ approximately 5% of nuclei were considered abnormal. Investigation of mutations for *TP53* (exons 2-11), *NOTCH1* (exon 34), *BIRC3* (exons 6-9), and *SF3B1* (exons 14-18) was performed by Next Generation Sequencing (NGS) approach assays with at least 1000X coverage and 1% sensitivity. Groups were compared by chi square test or *t*-test; OS and TTFT were computed from diagnosis to treatment/death or censored at last observation, and analyzed by log-rank test and Cox regression analysis. Details on the methods employed are reported elsewhere.[10,11]

In accordance with the Döhner categorization, cases with del17p (n=25) or del11q (n=24) were excluded from the whole series of tris12 CLL.[3] Moreover, to assess the impact of tris12 independent of *TP53* disruption, this latter being a well-established poor prognosticator,[11,12] cases bearing *TP53* abnormalities were further excluded from both the tris12 and control series (27 and 24 cases, respectively). The final dataset included 851 CLL, with 322 tris12 and 529 control cases (Figure 1A).

In the tris12 CLL series, the anomaly was found either as an isolated aberration in 237 cases (74%) or associated with del13q in 85 cases (26%; mosaic cases). In mosaic cases, the burden of each aberration, evaluated as the percentage of nuclei bearing tris12 and/or del13q (avail-

Table 1. Cox regression analysis.

	Trisomy 12 series				Control series[§]			
	UV		MV[§]		UV		MV[§]	
	HR	*P*	HR	*P*	HR	*P*	HR	*P*
OS								
CD49d pos	1.60	0.25	–	–	2.80	0.0001	2.29	0.0011
CD38 pos	1.38	0.28	–	–	2.39	0.0014	ns	ns
ZAP-70 pos	1.48	0.22	–	–	0.89	0.66	–	–
IGHV unmutated	2.13	0.0112	2.13	0.0112	2.23	0.0020	1.85	0.0202
NOTCH1 mutated	1.54	0.17	–	–	3.51	<0.0001	ns	ns
SF3B1 mutated	2.18	0.07	ns	ns	2.23	0.0329	ns	ns
BIRC3 mutated	1.46	0.23	–	–	2.06	0.32	–	–
TTFT								
CD49d pos	2.18	0.0006	2.20	0.0008	2.21	<0.0001	1.50	0.0247
CD38 pos	1.20	0.21	–	–	2.10	<0.0001	ns	ns
ZAP-70 pos	1.17	0.30	–	–	1.88	<0.0002	ns	ns
IGHV unmutated	1.67	0.0003	1.58	0.0014	4.38	<0.0001	3.17	<0.0001
NOTCH1 mutated	1.53	0.0046	ns	ns	4.51	<0.0001	2.13	0.0035
SF3B1 mutated	1.56	0.09	ns	ns	3.08	<0.0001	2.57	0.0003
BIRC3 mutated	1.10	0.55	–	–	3.32	<0.0084	ns	ns

[§]factors with *P*≤ 0.10 were entered in MV analysis. [§]del13q or negative FISH for del17p, del11q, trisomy 12, del13q. UV: univariate analysis; MV: multivariate analysis; HR: hazard ratio; *P*: log-rank test; OS: overall survival; TTFT: time to first treatment; Zap-70: Zeta-chain-associated protein kinase 70; ns: not significant factors after stepwise selection; pos: positive.

able in 66/85 cases), appeared randomly distributed (*Online Supplementary Table S2*). Mosaic cases did not show a greater mean age than isolated cases (64.5 years *vs.* 65.6 years, *P*=0.50, *t*-test). Both these findings are in favor of a random independent occurrence of these aberrations,[1] even if this hypothesis should be confirmed in sequential samples followed over time. Of note, upon normalizing the percentage of nuclei with tris12 by the disease load (i.e., the percentage of cells co-expressing CD5 and CD19 by flow cytometry), the median clonal prevalence of tris12-bearing nuclei was 94% within the malignant population (*data not shown*), in keeping with the hypothesized role of tris12 as an early and clonal event in CLL pathogenesis.[12]

When comparing tris12 CLL and the control series (*Online Supplementary Table S3*), no differences were found in median age, sex and Rai stage distribution. Median follow-up was 3.5 years (range 0-22) and 7.3 years (range 0-29) in tris12 CLL and the control series, respectively (*Online Supplementary Table S3*). Tris12 CLL had a higher prevalence of cases expressing CD49d, CD38, and cases with UM *IGHV, NOTCH1* mutations and *BIRC3* mutations (*Online Supplementary Table S3* and Figure 1B). Conversely, the frequency of *SF3B1* mutations and high ZAP-70 expression was not different between the two series (*Online Supplementary Table S3* and Figure 1B).

Details of *NOTCH1, BIRC3 and SF3B1* mutations are reported in *Online Supplementary Table S4*. Overall, we found 175 mutations in 148 tris12 cases (*Online Supplementary Table S4*). As previously reported, the vast majority of *NOTCH1* mutations (79/87) were disrupting mutations of the proline, glutamic acid, serine and threonine (PEST) domain;[11] similarly *BIRC3* mutations were mostly frameshift truncating mutations (62/70) while the 21 *SF3B1* mutations were either missense or in-frame deleting mutations. In all cases, missense mutations were validated as true somatic mutations by parallel analysis of sorted T cells (*data not shown*). Regarding the mutational load, *NOTCH1* mutations were well represented in the malignant clone, with 45/87 mutated cases (52%) demonstrating mutations in > 20% of DNA. Conversely, *BIRC3* and *SF3B1* mutations were predominantly subclonal (58/70 and 14/21 in less than 20% of DNA, respectively) in keeping with previous reports[12] (*Online Supplementary Table S4*).

Consistent with an intermediate prognosis under the Döhner classification, tris12 CLL patients experienced shorter OS and TTFT compared to the whole control series (Figure 2A,D), or separately to del13q- or normal karyotype-bearing cases (*Online Supplementary Figure S1*). Median OS was 15.6 years (tris12 CLL) and not reached (control series) with an overall mortality of 17% and 14%, respectively; median TTFT was 3.1 years (tris12 CLL) *versus* 14.9 years (control series), concerning 67% and 35% of treated patients, respectively. Previous authors have demonstrated that a high proportion of cells bearing tris12 is associated with a worse outcome.[3,13] In the series herein, we could not confirm this feature, neither with tris12 evaluated as a continuous variable, nor as a dichotomous variable applying the cut-off point at 60% of tris12-bearing nuclei, as previously suggested.[13]

Evaluating the clinical impact of the main CLL prognosticators in the tris12 CLL setting, we unexpectedly found that only the *IGHV* mutational status consistently correlated to both OS and TTFT (Table 1, Figure 2B,E), while all the other markers failed to reach statistical significance or, as with CD49d, impacted solely on TTFT (Table 1). An explorative analysis with alternative cut-off points for CD38, ZAP-70 and CD49d expression failed to improve their prognostic significance (*Online Supplementary Table S5*). Although *BIRC3* mutations had a rather high incidence in the tris12 series, comparable to that found in fludarabine refractory patients,[14] no statistical significance was reached, even when the cut-off point mutation level was altered from 1% to 10% to 20% (*data not shown*).

The prognostic relevance of *IGHV* mutational status in the tris12 series was confirmed by the application of the chronic lymphocytic leukemia international prognostic index (CLL-IPI) score, which contains the *IGHV* mutational status,[15] and was able to stratify both OS and TTFT (Figure 2C,F). Moreover, the small group of patients with mutated *IGHV* who expressed CD49d below the established cut-off point (8% of cases in our tris12 series), experienced longer TTFT intervals, almost resembling those of the control series (*Online Supplementary Figure S2*).

As opposed to tris12 cases, in univariate analysis carried out in the control series, CD49d, CD38, *IGHV* muta-

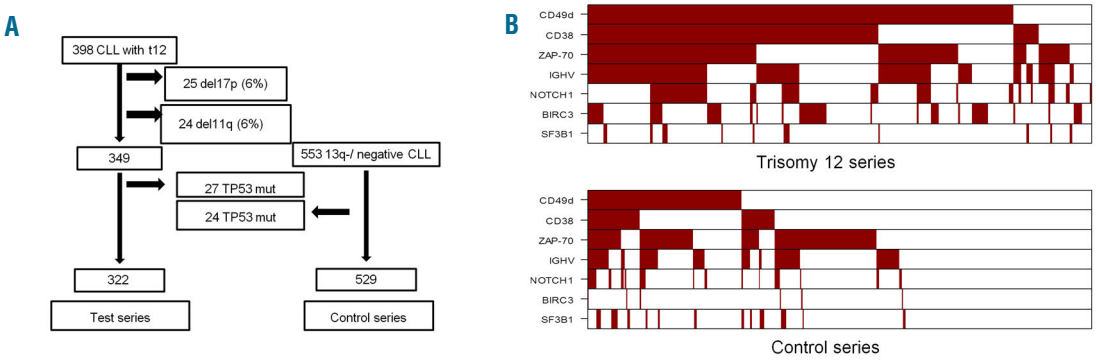

Figure 1. Selection of cases and distribution of biological prognosticators in trisomy 12 series and control series. (A) CONSORT flow diagram; B) Distribution among trisomy 12 (upper) and control series (lower) of the biological prognosticators CD49d expression, CD38 expression, ZAP-70 expression, *IGHV* mutational status, and mutations of *NOTCH1, BIRC3, SF3B1* genes, treated as dichotomous. Rows define the different prognosticators, columns define individual CLL cases. For each case, the presence of a defined feature (CD49d^{high}, CD38^{high}, ZAP-70^{high}, UM *IGHV, NOTCH1, BIRC3, SF3B1* mutations) is reported in dark red, the absence of the same feature is reported in white. CLL: Chronic Lymphocytic Leukemia; t12: trisomy 12; mut: mutated; Zap-70: Zeta-chain-associated protein kinase 70.

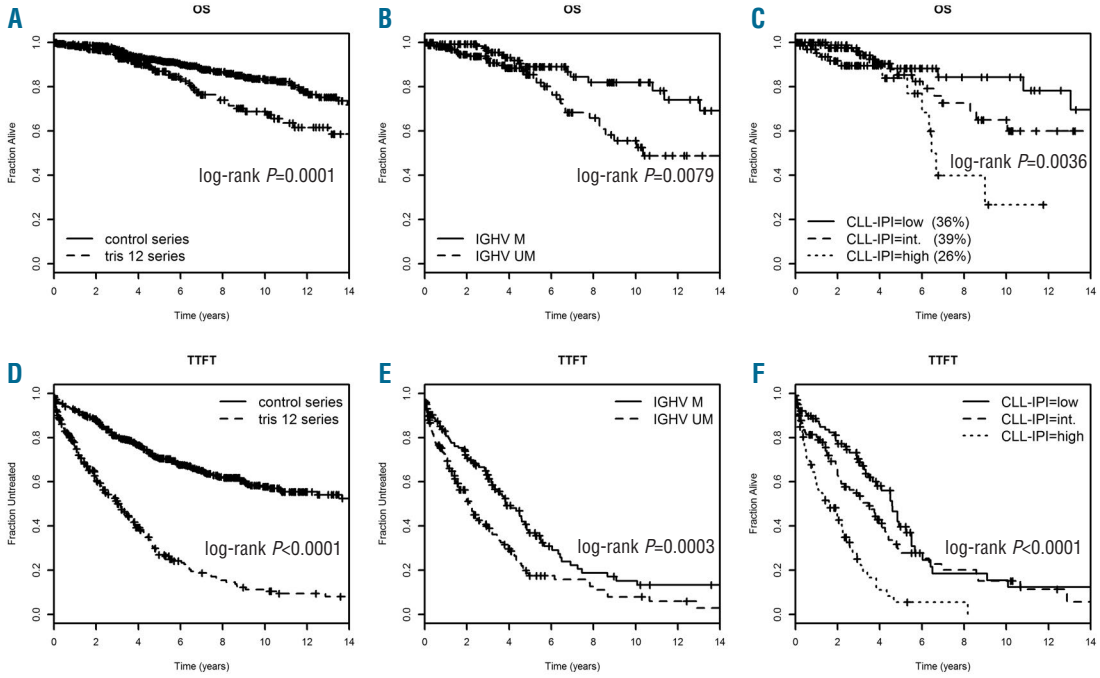

Figure 2. Kaplan-Meier curves and log-rank statistics comparing overall survival (OS) and time to first treatment (TTFT) in trisomy 12 and control series and stratification by *IGHV* mutations and The International Prognostic Index for patients with Chronic Lymphocytic Leukemia (CLL-IPI) score. (A) OS in trisomy 12 and control series; B) OS stratification in trisomy 12 series by IGHV mutations and C) by CLL-IPI score; D) TTFT in trisomy 12 and control series; E) TTFT stratification in trisomy 12 series by IGHV mutations and F) by CLL-IPI score.

tional status, *NOTCH1* and *SF3B1* were all associated to an inferior OS, as previously reported,[11] and all the variables, including ZAP-70 and *BIRC3*, associated with shorter TTFT intervals (Table 1). In multivariate analysis, both *IGHV* mutational status and CD49d expression proved to be independent prognosticators of shorter OS and TTFT, along with *NOTCH1* and *SF3B1* in TTFT analyses (Table 1), in keeping with previous studies.[11]

In summary, in the report herein, we demonstrated that the *IGHV* mutational status was the sole prognostic factor able to stratify OS and TTFT in tris12 CLL. Despite the high frequency of *NOTCH1* and *BIRC3* mutations and of CD49d and CD38 overexpression, these markers failed to convey a prognostic risk in this CLL subset. The peculiar clinical relevance of *IGHV* mutations in tris12 CLL might be related to a different pathobiology occurring in this subset, mainly involving B cell receptor-related microenvironment-driven pro-survival mechanisms rather than mechanisms ascribed to genetic lesions,[12] such as del13q, typically determining CLL cell survival *via* upregulation of the BCL2 antiapoptotic protein. Albeit the lack of independent clinical impact of *NOTCH1* and *SF3B1* mutations need to be confirmed in a prospective series, these findings add to other clinical and phenotypic peculiarities of tris12 CLL,[4,8] thus making a distinction between this CLL subset and the classical CLL.

Another observation of the study herein was that of the high prevalence of tris12-bearing nuclei, the elevated number of cases with isolated tris12, and the random occurrence of tris12/del13q association. Taken together, these findings are consistent with assigning to tris12 a role as a founder genetic lesion occurring early in CLL evolution in some patients.[12]

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