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Additional trisomies amongst patients with chronic lymphocytic leukemia carrying trisomy 12: the accompanying chromosome makes a difference

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Conflict of interest

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Recurrent cytogenetic abnormalities in chronic lymphocytic leukemia (CLL), namely deletions of chromosomes 11q, 13q, 17p and trisomy 12 (+12), define subgroups of patients with different clinical behavior and response to treatment[1]. We and others previously reported a minor fraction of CLL cases with co-existing trisomies of chromosomes 12 and 19 who share specific clinicobiological characteristics[2-4]. However, the cohort was small, thus precluding definitive conclusions. Here, taking advantage of a large, multi-institutional series, we confirm and significantly extend previous observations through the identification of subgroups of +12 CLL cases harboring particular concurrent trisomies demonstrating distinctive clinicobiological profiles.

In an unselected cohort of 4486 CLL patients with available classic cytogenetic (n=4285) or high-density 250K single nucleotide polymorphism (SNP)-array data (n=201) we identified 712 cases (16% of the cohort) carrying +12[5]. The median time from diagnosis to cytogenetic/SNP analysis was 1.5 months (range, 0-194); the majority of cases included in survival analysis were untreated prior to testing (94%). The study was approved by the local Ethics Review Committees. Details about the study cohort and the methodologies utilized are provided in the Supplementary Material.

Of the 712 +12 CLL cases, 86 (12% or 2% of the entire cohort) harbored multiple trisomies; 68/86 cases (78% or 1.5% of the entire cohort) had co-existing +19 (+12+19 CLL), while the remaining 18/86 cases (22% or 0.5% of the entire cohort) were negative for +19 and instead carried other co-existing extra chromosome(s) (+12,+other-non19 CLL) (Supplemental Table 1, Supplemental Figure 1).

Amongst +12+19 cases, 49/68 (72%) harbored additional numerical and/or structural aberrations. Trisomy 18 was the predominant co-existing abnormality and was detected in 42/68 cases (62%) (Figure 1A). Comparison of +12+19 cases with/without +18 revealed no significant differences (regarding age and stage at diagnosis, gender, IGHV mutational status, CD38 expression and clinical outcome, data not shown), suggesting that +18 might represent a secondary event, probably related to clonal evolution. This claim is also supported by our unpublished FISH data using chromosome 12 and 18 centromeric probes in cases with +12+18+19, revealing cells with +12 alone, cells with +12+18 but not cells with +18 alone. Additional structural abnormalities, primarily concerning chromosome 13q, were observed in 12/63 (18%) cases, of whom 10 also carried +18. Only 1/23 (4%) and 3/59 (5%) cases with

available data carried mutations in the *NOTCH1* and *TP53* genes, respectively[6, 7]; none of these cases carried +18.

The +12+19 CLL subgroup concerned relatively young patients (median age at diagnosis: 59 years). In keeping with our previous report, all +12+19 CLL cases with available data (n=23) expressed surface IgG. Considering the low frequency of CLL cases with switched immunoglobulin (IG) in their B-cell receptors (BcR), this finding is highly suggestive of a particular immunopathogenetic process in this patient subgroup[8]. This claim was further supported by the remarkable bias to lambda light chain expression in this particular cytogenetic subgroup [22/32 cases (69%) with available data], raising the intriguing possibility that the respective clonogenic progenitors may have been subject to light chain receptor editing[9, 10].

All but one +12+19 CLL case with available information (47/48, 98%) carried immunoglobulin heavy variable (IGHV) genes impacted to some degree by somatic hypermutation (SHM), leading to IGHV genes with <100% germline identity. Following the 98% germline identity cut-off value, only 2/48 (4%) cases were classified as IG-unmutated CLL (U-CLL), whereas the remaining 46 carried numerous SHMs and were classified as IG-mutated CLL (M-CLL). Despite this, in keeping with our previous study, the majority of +12+19 cases (41/60, 68%) expressed CD38[2]. Unexpectedly, 23/37 (62%) +12+19 cases were found to express monoclonal paraprotein detectable by immunofixation electrophoresis with identical heavy (gamma) and light chain to that present on the surface of the circulating CLL cells. This is significantly higher compared to generic CLL cohorts and merits further investigation[11], especially since paraproteinemia in CLL has not thus far been associated with a particular cytogenetic aberration. Of note, amongst +12+19 cases, no differences were identified regarding other clinicobiological features, including CD38 positivity, between cases with/without paraproteinemia.

Turning to the +12+other-non+19 CLL subgroup, the co-existing cytogenetic aberrations were exclusively numerical: trisomy 3 predominated (7/18 cases, 39%), followed by trisomies 18 and 22 in 6 (33%) and 4 (22%) cases, respectively (Figure 1B). Similar to what was reported above, most cases (7/9, 78%) belonged to the M-CLL category and expressed CD38 (11/17 cases, 65%). Recurrent gene mutations were very rare, indeed only 1/13 (8%) patients carried a *TP53* mutation, whereas no *NOTCH1* mutations were identified[6].

Perhaps the most notable features of the +12+other-non+19 subgroup concerned the high incidence of clinical/laboratory autoimmune manifestations[12] (4/9 cases with available information, 45%) and other malignancies[13] (5/10 cases with additional information, 50%). Regarding the former, these included: autoimmune hemolytic anemia (n=2) as well as a positive direct antiglobulin (n=1) and rheumatoid factor test (n=1); 3/5 of these cases never required treatment for their CLL. Other co-existing malignancies concerned: prostate cancer (n=2), bladder cancer (n=1), adenocarcinoma of the uterus (n=1) and basal cell carcinoma of the skin (n=1); one of these 5 cases never received any treatment for CLL, while among the remaining 4, three were diagnosed with the other malignancy prior to receiving any CLL-specific treatment. Altogether, the link with malignancy identified in our study is intriguing, however it requires further investigation in larger patient cohorts.

Comparative assessment of the two subgroups reported above, namely +12+19 CLL and +12+other-non+19 CLL, revealed significant differences regarding heavy IgG-isotype expression ($p<0.0001$) as well as the incidence of del(13q) ($p=0.001$), autoimmune manifestations ($p=0.013$) and second malignancies ($p=0.018$) (Table 1). Comparisons were also made to 65 cases of the present series carrying isolated +12 as detected by classic cytogenetic analysis, illustrating that the two subgroups reported here display distinct subgroup-biased profiles (Table 1). In particular, both subgroups were enriched for M-CLL compared to isolated +12 CLL ($p<0.001$ and $p=0.032$ for +12+19 and +12+other-non+19, respectively). The +12+19 CLL subgroup also differed significantly from isolated +12 cases regarding age at diagnosis (younger amongst the former), higher incidence of IgG-switched heavy and lambda light chains, CD38 positivity as well as higher incidence of co-existing del(13q) ($p=0.001$). Moving on to the +12+other-non+19 CLL subgroup, comparison to isolated +12 cases disclosed a significantly higher incidence of autoimmune manifestations and other malignancies. Finally, both subgroups experienced a more indolent clinical course compared to cases with isolated +12, reflected in a significantly longer overall survival (OS). In fact, whereas a median OS of 7.9 years was observed for cases with isolated +12, the median OS for +12+other-non+19 subgroup was 16 years while that of the +12+19 subgroup had not yet been reached ($p=0.033$ and $p=0.013$, respectively). These findings corroborate previous reports that cytogenetic complexity defined by solely numerical aberrations within CLL should not be axiomatically considered as an unfavorable prognostic marker. That said, it

should be acknowledged that the favorable clinical outcome within +12,+19 and +12,+other-non19 subgroups might be attributed to their enrichment for M-CLL (Supplemental Figure 2). Amongst various host- and tumor-related parameters assessed for their prognostic/predictive relevance, cytogenetic aberrations and recurrent gene mutations have attracted the greatest interest[7, 14, 15]. Trisomy 12 is the second most frequent recurrent chromosomal aberration in CLL and is associated with clinical and biological heterogeneity, potentially linked to the presence of additional genomic aberrations. This concept is reinforced by our present findings regarding the biological background and clinical presentation/outcome of subgroups of +12 CLL patients defined by the presence of extra trisomies. These subgroups seem to differ from patients with isolated +12 while also exhibiting a constellation of biological and clinical features whose co-occurrence is rather unlikely to be fortuitous. This conclusion is also supported by our query for CLL cases with trisomy 19 in the Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (http://cgap.nci.nih.gov/Chromosomes/Mitelman) which retrieved 66 cases of which 56 (85%) displayed a co-existing +12. Taking all the above into consideration, +19 in CLL appears to be heavily biased to patients carrying +12, alluding to a unique pathway of clonal evolution. As for the +12+other-non+19 subgroup, although caution is warranted due to the low number of cases, the lack of any structural chromosomal aberration and paucity of recurrent gene mutations is noteworthy.

In conclusion, we report the existence of subgroups within +12 CLL defined by the presence of extra trisomies, associated with subgroup-biased profiles of potential clinical relevance. The biological mechanisms underlying both the acquisition of additional chromosomes and, particularly, the specific phenotypes of these subgroups remain to be elucidated.

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Table 1. Comparison of +12+19 versus +12+other-non+19 versus isolated +12 cases. Due to multiple testing, the Bonferroni correction is applied per line and each individual hypothesis is tested at a statistical significance level of 0.0167.

	A: +12+19 n=68	B: +12+ other-non+19 n=18	C: isolated +12 n=65	p-value A vs B	p-value A vs C	p-value B vs C
Male gender	52/68 (76%)	12/18 (67%)	43/65 (66%)	0.382	0.249	1.000
Median age at diagnosis (years)	59	67	67	0.007	0.0001	0.76
<55 years	24/65 (37%)	0/14 (0%)	2/57 (4%)	0.031	<0.0001	0.509
Binet A	50/61 (82%)	14/17(82%)	47/57 (82%)	1.000	1.000	1.000
Binet B	5/61 (8%)	2/17 (12%)	8/57 (14%)	0.642	0.384	1.000
Binet C	6/61 (10%)	1/17 (6%)	2/57 (4%)	1.000	0.274	0.586
M-CLL[1]	46/48 (96%)	7/9 (78%)	23/61 (38%)	0.113	<0.0001	0.032
IgG isotype	23/23 (100%)	1/8 (12%)	8/45 (18%)	<0.0001	<0.0001	1.000
Lambda light chain expression	22/32 (69%)	2/8 (25%)	14/46 (30%)	0.042	0.001	1.000
CD38 expression[2]	41/60 (67%)	11/17 (65%)	25/56[3] (45%)	0.776	0.014	0.814
del(13q)	27/51 (53%)	0/11 (0%)	13/48[4] (27%)	0.001	0.014	0.100
del(11q)	0/53 (0%)	0/10 (0%)	1/47 (2%)	1.000	0.47	1.000
TP53abn[5]	3/59 (5%)	1/13 (8%)	4/56 (7%)	0.557	0.712	1.000
NOTCH1	1/23 (4%)	0/7 (0%)	4/37[6] (11%)	1.000	0.640	1.000
IG spike	23/37 (62%)	2/8 (25%)	No data	0.113	-	-
Autoimmunity	2/33 (6%)	4/9 (50%)	0/22 (0%)	0.013	0.511	0.004
Other malignancy	4/48 (8%)	5/10 (55%)	4/43 (9%)	0.018	1.000	0.042

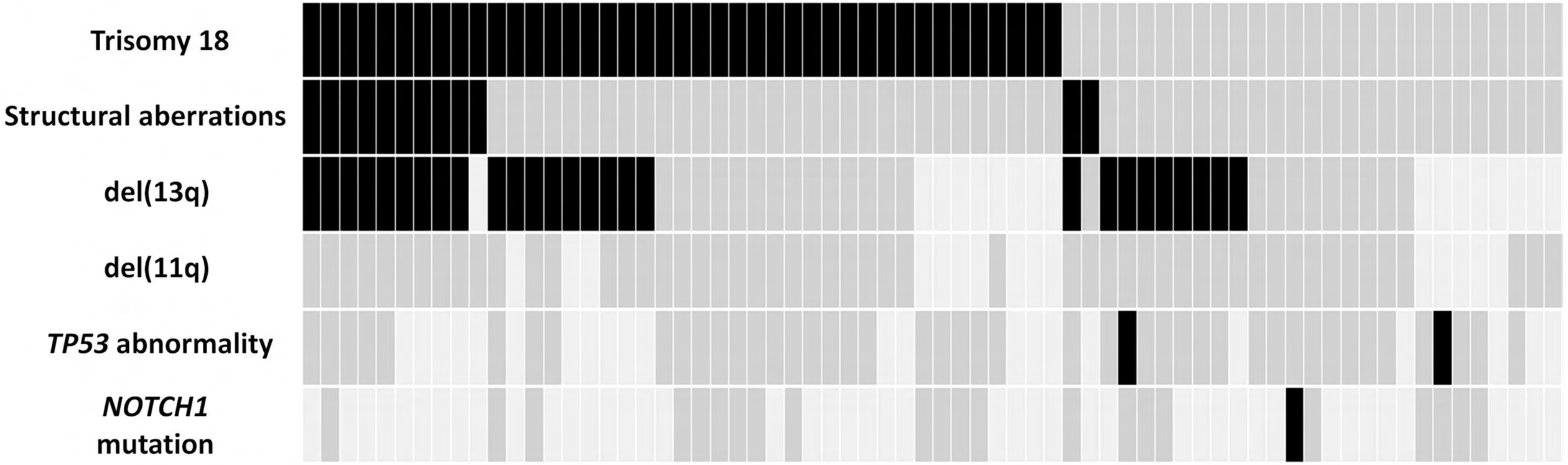
1: CLL with mutated IGHV genes; 2: cut-off for positivity: 30%; 3: amongst +12/M-CLL, CD38 high expression: 3/17 (19%); 4: amongst +12/M-CLL, del(13q): 8/16 (50%); 5: *TP53abn*: deletion of chromosome 17p and/or *TP53* mutation; 6: amongst +12/M-CLL, NOTCH1: 0/15 (0%)

Figure legends

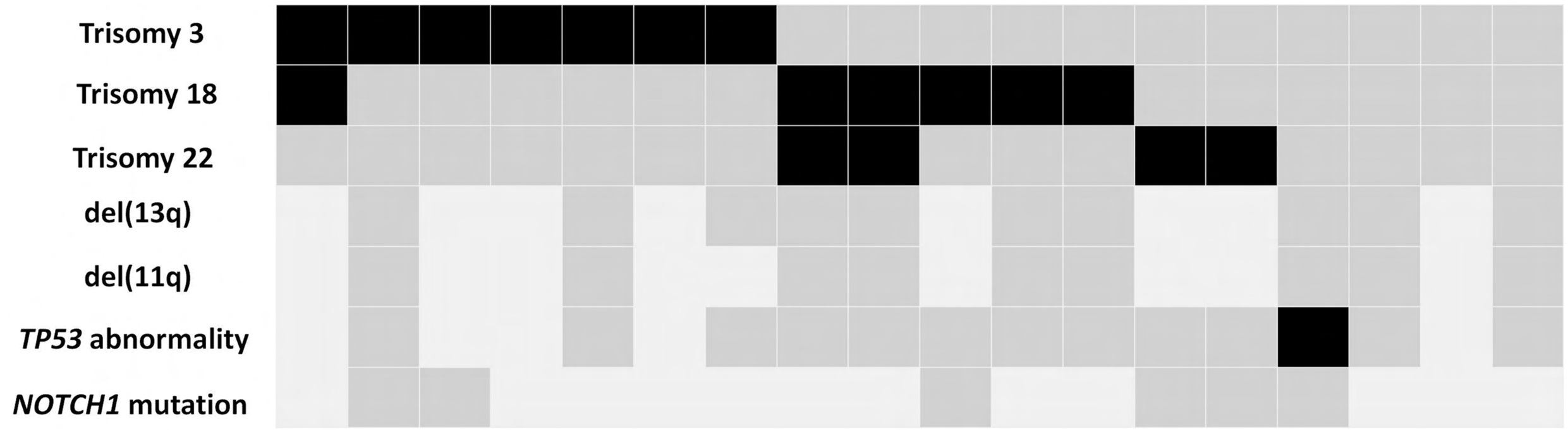
Figure 1. Genomic profile of: (A) +12,+19 and (B) +12,+other-non+19 CLL. The rows correspond to the specific lesion indicated while the columns represent individual patients. Colored: positive (black), negative (dark grey), no data (light grey). del(13q): deletion of chromosome 13q detected by FISH, del(11q): deletion of chromosome 11q detected by FISH; *TP53* abnormality: deletion of chromosome 17p and/or *TP53* mutation.

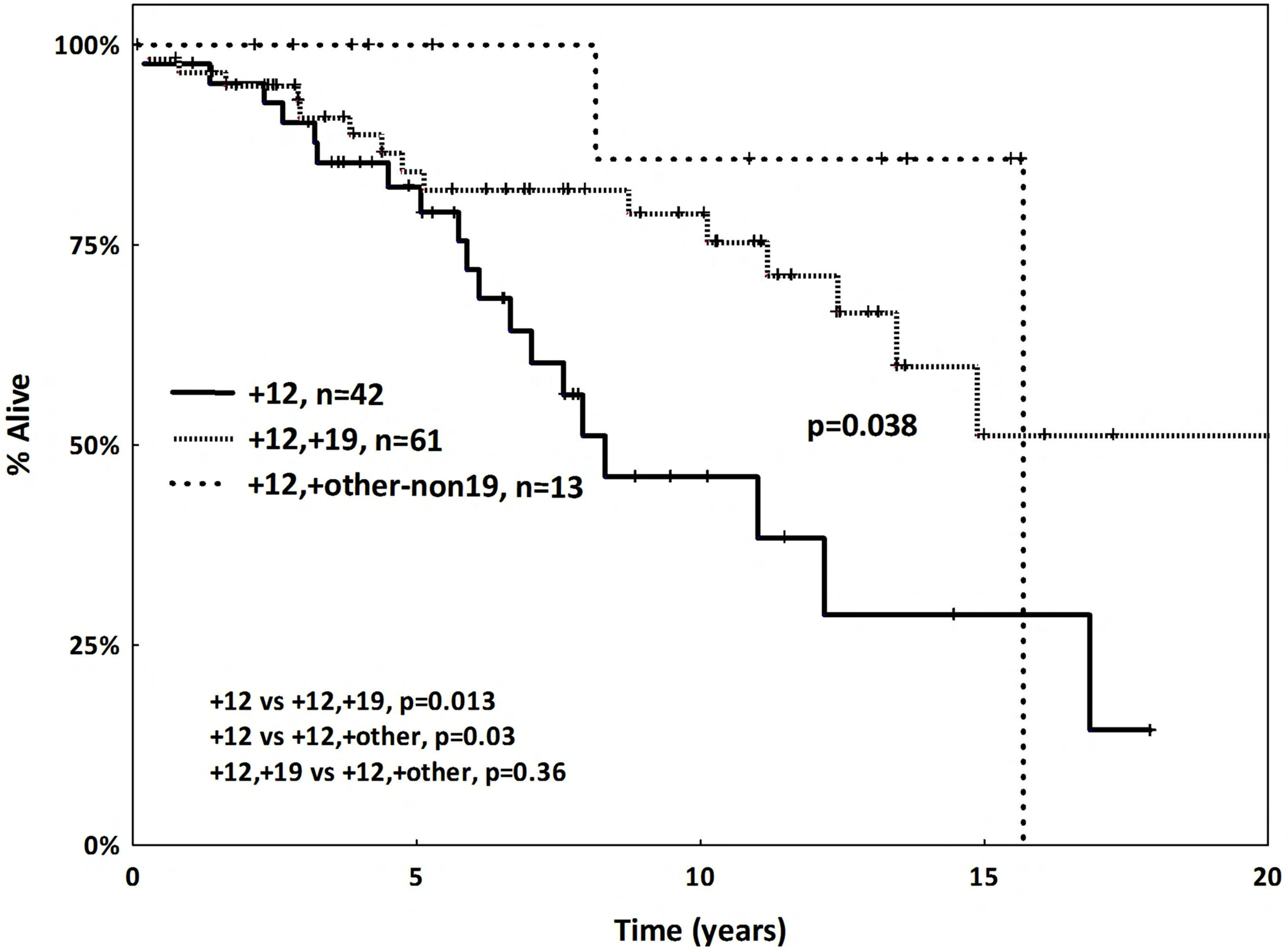
Figure 2. Kaplan-Meier curves for overall survival (OS). The +12,+19 and +12+other-non19 CLL cases demonstrated significantly prolonged OS compared to CLL cases with isolated +12 on classic cytogenetic analysis.

A


Trisomy 18
Structural aberrations
del(13q)
del(11q)
TP53 abnormality
NOTCH1 mutation

B


Trisomy 3
Trisomy 18
Trisomy 22
del(13q)
del(11q)
TP53 abnormality
NOTCH1 mutation


100%
75%
50%
25%
0%
% Alive
+12, n=42
+12,+19, n=61
+12,+other-non19, n=13
p=0.038
+12 vs +12,+19, p=0.013
+12 vs +12,+other, p=0.03
+12,+19 vs +12,+other, p=0.36
0
5
10
15
20
Time (years)

Supplementary material

Additional trisomies amongst patients with chronic lymphocytic leukemia carrying trisomy 12: the accompanying chromosome makes a difference

The Supplementary material contains information regarding the patient series and the methodologies utilized.

Classic Cytogenetic and FISH analysis

For metaphase induction, 10^6 per mL peripheral blood mononuclear cells were cultured using two different protocols. More specifically, until 2008, cells were cultured for 72 and 96 hours in RPMI 1640 medium with 20% fetal calf serum and phorbol-12-myristate-13-acetate (TPA) at 50ng/mL; colcemid (0.01 μg/ml) was added 45 min before harvest. Since 2008, metaphases were obtained after culturing in RPMI 1640 medium with 20% fetal calf serum in the presence of the immunostimulatory CpG-oligonucleotide DSP30 and interleukin 2 (IL-2) (200 U/mL); after 48 hours, colcemid (0.015 μg/ml), was added for an additional 24 hours prior to the preparation of chromosomes. Hypotonic treatment was performed using 0.075 M KCl, and fixation of the chromosomes was accomplished using a methanol:glacial acetic acid (3:1) fixative solution. Chromosome preparation and staining was performed according to standard protocols. A minimum of 15 mitotic cells were examined and karyotypes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN) 2009. Karyotypes obtained before 2009, were re-classified following the ISCN from 2013.
Interphase *fluorescence in situ hybridization* (FISH) analysis was conducted using probes to detect the recurrent genomic aberrations in CLL. More specifically probes were used to detect (del)11q23 (LSI ATM and CEP11), del(13q14) (D13S319 and LSI13q34) for del(17p13) (LSI TP53 and CEP17). For trisomy 12 only CEP12 was used.

Microarray single nucleotide polymorphism analysis (250K Affymetrix arrays)

SNP-array experiments were performed according to the standard protocols for Affymetrix GeneChip® Mapping NspI-250K arrays and as previously described[1].

Analysis of gene mutations

Mutational screening was performed for the following genes: *NOTCH1*: entire exon 34 or targeted analysis for del7544-45/p.P2514Rfs*4; *TP53*: exons 4-8 as previously described[2].

CD38 expression

CD38 expression was assessed with flow-cytometry (threshold for positivity: 30%).

PCR amplification and sequence analysis of IGHV-IGHD-IGHJ rearrangements

PCR amplification and sequence analysis of IGHV-IGHD-IGHJ rearrangements were performed on either genomic DNA (gDNA) or complementary DNA (cDNA), as previously described or following the BIOMED-2 protocol[3]. PCR amplicons were subjected to direct sequencing on both strands. Sequence data were analyzed using the IMGT® databases and the IMGT/V-QUEST tool (http://www.imgt.org). Only productive rearrangements were evaluated. Output data from IMGT/V-QUEST for all productive IGHV-IGHD-IGHJ rearrangements were parsed, reorganized, and exported to a spreadsheet through the use of computer programming. Information was extracted regarding IG gene repertoires, VH CDR3 length and amino acid sequence and the % germline identity.

Statistical analysis

Descriptive analysis included frequency distributions for all the categorical variables. Associations regarding categorical variables were assessed using the Chi-square or Fisher's exact test for independence, in case the evaluated group included less than 20 cases. Overall survival (OS) was calculated using the date of diagnosis and the date of death or last follow-up. Kaplan Meier survival analysis and the log-rank test were used to assess differences in OS

between patient subgroups for different variables. All tests were two sided and significance was defined as a p value less than 0.05. Statistical analysis was performed using the Statistica Software 10·0 (StatSoftInc, Tulsa,OK) and R-3.2.1 programming language.

Supplemental Table 1. +12 CLL carrying additional trisomies.

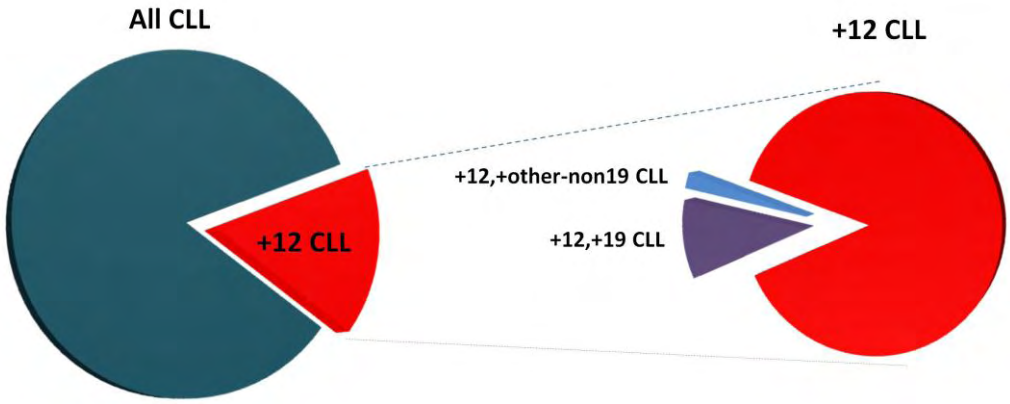
Case	Classic cytogenetic analysis
+12+19 CLL	
1	48,XY,+12,+19[16]
2	49,XY,+12,+18,+19[4]/49,XY,idem,del(13)(q14)[2]/46,XY[10]
3	49,XX,+12,+18,+19[18]
4	48,XY,+12,+19[18]
5	48,XY,+12,+19[5]/46,XY[10]
6	50,XY,+12,+18,+19,+22[2]/46,XY[18]
7	50,XY,+12,+18,+19,+22[2]/46,XY[38]
8	49,XY,+12,+18,+19[4]/46,XY[16]
9	49,XY,+12,+18,+19[6]/46,XY[14]
10	50,XY,+8,+12,+18,+19 [13]/50,idem,?del(3)(p2?2)[2]
11	49,XY,+12,+18,+19[18]/46,XY[2]
12	47,X,-Y,+12,+19[6]/46,XY[14]
13	48,XX,+12,+19[20]
14	49,XX,+12,+19,+22[20]
15	48,XY,+12,+18[3]/49,idem,+19[11]/46,XY[7]
16	49,+12,+18,+19[15]/46,XY[4]
17	48,XY,+12,+19[6]/46,XY[13]
18	48,XY,+12,+19[4]/48,idem,t(6;14)(p2?3;q2?3)[6]/46,XY[14]
19	50,XY,+12,+18,+19,+22[6]/46,XY[14]
20	49,XY,+12,+18,+19[8]/46,XY[12]
21	49,XY,+12,+18,+19[15]/46,XY[5]
22	49,XY,+12,+18,+19[6]/46,XY[14]
23	49,XY,+12,+18,+19[10]/46,XY[9]
24	48,XY,+12,+19[8]/46,XY[12]
25	47,XY,+12[2]/48,idem,+19[14]/46,XY[4]
26	+12,+19*[1]
27	+12,+18,+19*[1]
28	+12,+18,+19*[1]
29	+12,+19*[1]
30	+12,+19*[1]
31	46,XY,del(13q14)[6]/49,XY,+12,+18,+19[4]/46,XY[3]
32	48,XY,+12,+19[9]/46,XY[11]
33	49,XY,+12,+18,+19[7]/46,XY[13]
34	49,XY,+12,+19,+mar[2]/46,XY[18]
35	49,XY,+12,+18,+19[6]/46,XY[9]
36	48,XX,+12,+19[17]/46,XX[20]

37	49,XX,+12,+18,+19[3]/46,XX[17]
38	49,XX,+12,+18,+19[5]/46,XX[15]
39	47,X,-X,+12,+19[7]/46,XX[13]
40	48,XY,+12,+19[15]/46,XY[5]
41	49,XY,+12,+18,+19[10]/46,XY[11]
42	48,XX,+12,+19[1]/46,XX[22]*[2]
43	49,XY,+12,+18,+19[6]/46,XY[9]
44	50,XY,+Y,t(10;10)(q25;q23),+12,+18, +19[4]/46,XY[12]
45	48,XY,+12,+19[3]/46,XY[17]
46	48,XX,+12,+19[3]/46,XY[17]
47	48,XY,+12,+19[3]/46,XY[17]
48	50,XY,+8,+12,del(13)(q11q14),t(13;14)(q14;q24),+18,+19[20]
49	49,XY,+12,+18,+19[14]/46,XY[6]
50	49,XY,+12,+18,+19[3]/46,XY[17]
51	49,XY,+12,+18,+19[8]46,XY[22]
52	49,XY,t(9;13)(q?32;q?14),+12,+18,+19 [15]/46,XY[3]
53	49,XX,+12,+18,+19[9]/46,XX[11]
54	49,XY,+12,+18,+19[17]/46,XY[3]
55	49,XY,+12,+18,+19,?del(9)(q?),?add(13)(q?)[7]/46,XY[13]
56	49,XX,+12,+18,+19[17]/46,XX[3]
57	49,XY,+12,+18,+19[20]
58	49,XY,+12,?del(13)(q14q22),+18,+19[8]/46,XY[12]
59	49,XY,+12,+18,+19 [9]49,idem,t(13;14)(q14;q24)[2]/46,XY[9]
60	48,XY,?11,t(12;13)(p11;q?12),+12+19[10]/46,XY[10]
61	49,XY,+12,+18,+19[8]/46,XY[12]
62	49,XX,+12,+18,+19[10]/46,XX[10]
63	48,XX,+12,+19[7]/46,XX[13]
64	48,XY,+12,+19[9]/46,XY[11]
65	48,XY,+12,+19[3]/46,XY[16]
66	49,XY,+12,+18,+19[20]
67	49,XY,+12,+13,+18,+19[17]/49,idem,del(17)(p11)[2]
68	48,XY,+12,+19[20]
+12+other-non19 CLL	
69	48,XX,+12,+21[7]/46,XX [13]
70	48,XY,+12,+22[6]/46,XY[14]
71	48,XY,+3,+12[9]/46,XY[11]
72	+12,+18*[1]
73	48,XY,+8,+12[10]/46,XY[10]
74	49,XY,+3,+12,+18[7]/46,XY[15]
75	47,X,-Y,+3,+12[3]/46,XY[17]

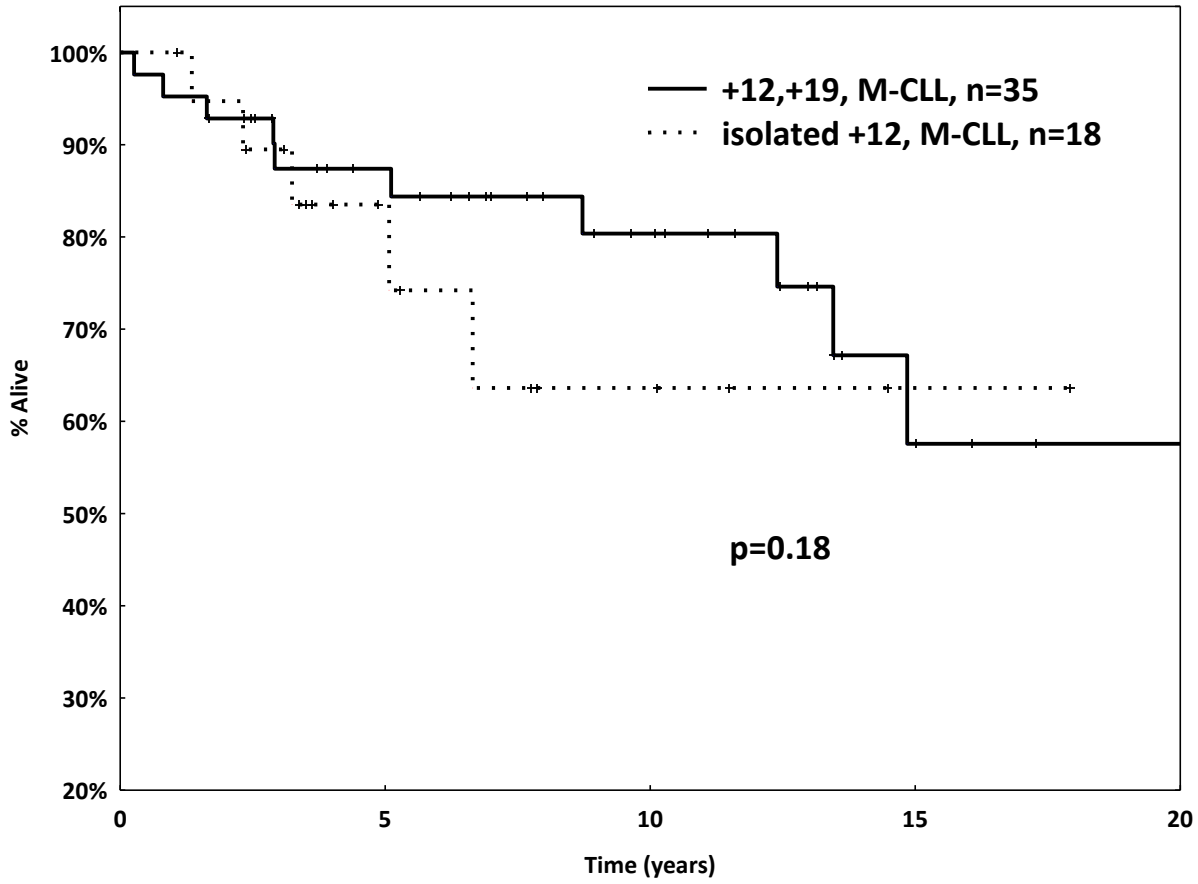
76	49,XY,+12,+18,+22[6]/46,XY[14]
77	48,XY,+3,+12[8]/46,XY[9]
78	48,XX,+3,+12[3]/46,XX[17]
79	48,XY,+3,+12[2]/46,XY[18]
80	48,XX,+12,+18 [4]46,XX[1]
81	49,XX,+12,+16,+18[7]/46,XX[22]
82	48,XY,+12,+21[22]/46,XY[7]
83	48,XY,+8,+12[16]/46,XY[6]
84	48,XY,+12,+22[6]/46,XY[15]
85	48,XY,+3,+12[9]/46,XY[11]
86	49,XY,+12,+18,+22[6]/46,XY[14]

*1: according to high-density 250K single nucleotide polymorphism analysis
*[2]: verified by FISH analysis

Supplemental Figure 1. Left: proportion of +12 CLL within the whole CLL cohort. Right: Proportion of +12,+19 and +12,+othet-non19 CLL within +12 CLL.



Supplemental Figure 2. Kaplan-Meier curves for overall survival (OS) within cases carrying mutated IGHV genes (M-CLL). No significant difference between cases with +12,+19 and those with isolated +12 is observed, however this could be due to low numbers.



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