Telomere dysfunction accurately predicts clinical outcome in chronic lymphocytic leukaemia even in patients with early stage disease

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Abstract

Defining the prognosis of individual cancer sufferers remains a significant clinical challenge. Here we assessed the ability of high-resolution single telomere length analysis (STELA), combined with an experimentally derived definition of telomere dysfunction, to predict the clinical outcome of patients with chronic lymphocytic leukaemia (CLL). We defined the upper telomere length threshold at which telomere fusions occur and then used the mean of the telomere 'fusogenic' range as a prognostic tool. Patients with telomeres within the fusogenic range had a significantly shorter overall survival (P<0·0001; HR = 13.2 (11.6-106.4)) and this was preserved in early-stage disease patients (P<0·0001, HR = 19.3 (17.8-802.5)). Indeed, our assay allowed the accurate stratification of Binet stage A patients into those with indolent disease (91% survival at 10 years) and those with poor prognosis (13% survival at 10 years). Furthermore, patients with telomeres above the fusogenic mean showed superior prognosis regardless of their *IGHV* mutation status or cytogenetic risk group. In keeping with this finding, telomere dysfunction was the dominant variable in multivariate analysis. Taken together, this study provides compelling evidence for the use of high-resolution telomere length analysis coupled with a definition of telomere dysfunction in the prognostic assessment of CLL.

Introduction

Diagnostic testing is now available for most common cancers. However, defining the prognosis of individual patients remains a significant clinical challenge. This is exemplified by the clinical conundrum that is chronic lymphocytic leukaemia (CLL); a disease with a heterogeneous clinical course ranging from a few months to many decades (Hallek 2010). Although the Binet and Rai staging systems are reliable predictors of outcome between the staging groups, they fail to identify good and bad prognostic subsets within each stage. Since most patients are diagnosed with early stage disease (Rozman*, et al* 1997), a number of laboratory tests have been developed to predict the clinical course of these patients most notably *IGHV* mutation status, CD38 expression, ZAP70 expression and cytogenetic abnormalities (Damle*, et al* 1999, Dohner*, et al* 1999, Hamblin*, et al* 1999, Rassenti*, et al* 2004). Unmutated *IGHV* genes, high CD38 expression, high ZAP70 expression and the presence of 17p and 11q deletions are all associated with a bad prognosis (Hamblin*, et al* 2002, Rassenti*, et al* 2004), but none of these individual markers can provide definitive prognostic information either alone or in combination. Consequently there is a need to identify prognostic tools with the capacity to provide personalised risk assessments.

Telomeres are nucleoprotein structures that cap the ends of linear eukaryotic chromosomes (de Lange 2005). Telomere length is a key determinant of telomeric function and short dysfunctional telomeres can drive genomic instability and tumourigenesis in mouse models (Artandi*, et al* 2000). There is now substantial evidence that telomere erosion and fusion plays an important role in the pathology of many common human malignancies including CLL (Damle*, et al* 2004, Gertler*, et al* 2004, Meeker and Argani 2004, Ricca*, et al* 2007, Roos*, et al* 2008, Rossi*, et al* 2009). However, the lack of resolution of the currently available technologies has hampered progress in translating telomeric assays into clinical practice. A

key problem with these technologies is their inability to detect short telomeres within the length ranges at which dysfunction and fusion occurs (Aubert*, et al* 2012, Baird 2005, Lin*, et al* 2010). To address this problem, we have developed single-molecule technologies that allow the detection of critically shortened telomeres (Baird*, et al* 2003, Britt-Compton*, et al* 2006) and the characterisation of telomere dysfunction (Capper*, et al* 2007, Letsolo*, et al* 2010). Using these tools, we have demonstrated a link between short telomeres, telomere fusion and genomic instability in CLL (Lin*, et al* 2010). Importantly we showed that a subset of early-stage patients exhibited extensive telomere erosion and fusion, indicating that telomere shortening and dysfunction can precede clinical progression. In this present study we defined, for the first time, the telomere length threshold at which fusion events occur and used the fusogenic mean as a prognostic tool in a cohort of 200 CLL patients and an independent validation cohort of 121 CLL patients.

Methods

CLL Patients

Peripheral blood samples from 200 CLL patients were obtained after written informed consent had been received in accordance with the Declaration of Helsinki and as approved by the South East Wales local research ethics committee (LREC# 02/4806). Patients were selected purely on a definitive diagnosis of CLL as defined by IWCLL criteria (Hallek*, et al* 2008). Comprehensive clinical information was available for all patients with a median follow-up of 5.6 years. All of the samples were collected at, or close to, the time of diagnosis (<6 months) from two centres, Cardiff and Birmingham, and staging was based on the Binet classification system (Binet*, et al* 1981). Time to first treatment was defined from date of diagnosis to date of first treatment intervention. Progression-free survival was calculated from date of diagnosis to the point where disease progressed to a more advanced Binet stage. Overall survival was quantified from date of diagnosis to date of death or date of last follow-up. The CLL patients' clinical/laboratory characteristics are summarised in Table S1.

CLL validation cohort

Purified DNA extracted from peripheral blood samples from 121 CLL patients was obtained from our collaborators in Hull managed by the Newcastle CLL consortium as part of the Newcastle Haematology Biobank (REC ref 07/H0906/109+5). Collection of blood samples and clinico-pathologic information from patients was undertaken with informed consent in accordance with the tenets of the Declaration of Helsinki. The CLL patients' clinical/laboratory characteristics are summarised in Table S2.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of CLL patients by density centrifugation using Ficoll-Hypaque (Invitrogen, Paisley, UK). For the discovery CLL cohort B-cells were subsequently positively isolated using CD19-labelled Dynabeads (Invitrogen, Paisley, UK) (Hewamana*, et al* 2008). For the CLL validation cohort and LRF CLL4 trial cohort, DNA was extracted from unselected PBMC. Cells were stored at -20°C as dry pellets prior to DNA extraction.

DNA extraction and single telomere length analysis

DNA was extracted from using standard proteinase K, RNase A, phenol/chloroform protocols or the QIAamp DNA Blood Mini Kit (Qiagen, Manchester, UK). For telomere length analysis at the XpYp telomere we used the single telomere length analysis (STELA) assay previously described (Baird*, et al* 2003, Britt-Compton*, et al* 2006, Capper*, et al* 2007). Briefly genomic DNA was solubilised by dilution in 10mM Tris-HCl PH7.5, quantified by Hoechst 33258 fluorometry (BioRad, Hercules, USA) and diluted to 10 ng/µl in 10mM Tris-HCl pH 7.5. 10 ng of DNA was further diluted to 250 pg/µl in a volume of 40µl containing 1µM Telorette2 linker and 1 mM Tris-HCl pH 7.5. Multiple PCRs (typically 6 reactions per sample) were carried out for each test DNA in 10µl volumes 250pg of DNA, 0.5 µM of the telomere-adjacent and Teltail primers, 75mM Tris-HCl pH 8.8, 20 mM $(NH4)_2SO_4$, 0.01% Tween-20, 1.5 mM $MgCl_2$, and 0.05 U of a 10:1 mixture of Taq (ABGene, Epsom, UK) and Pwo polymerase (Roche Molecular Biochemicals, Lewes, UK). The reactions were cycled with a Tetrad2 thermocycler (BioRad, USA) under the following conditions: 22 cycles of 94^{o}C for 15 seconds, 65^{o}C for 30 seconds and 68^{o}C for 8mins. The DNA fragments were resolved by 0.5% TAE agarose gel electrophoresis, and detected by Southern hybridisations with random-primed α-33P labelled (Ready-To-Go DNA Labeling Beads, GE Healthcare,

Little Chalfont, UK) TTAGGG repeat probe, together with a probe to detect the 1 kb (Stratagene, La Jolla, USA) and 2.5 kb (BioRad) molecular weight marker. The hybridised fragments were detected by phosphorimaging with a Typhoon phosphorimager (GE Healthcare, USA). The molecular weights of the DNA fragments were calculated using the Phoretix 1D quantifier (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

Telomere fusion assay

The telomere fusion assay was carried out as described previously (Capper*, et al* 2007, Letsolo*, et al* 2010). PCR reactions were carried out each containing 100 ng of DNA with XpYpM, 17p6 and 21q1 PCR primers (Letsolo*, et al* 2010). Fusion molecules were detected by Southern blotting and hybridisation with the XpYp, 17p and 21q telomere-adjacent probes as described previously (Capper*, et al* 2007, Letsolo*, et al* 2010). Any fusion products were then re-amplified for direct sequence analysis using nested PCR primers (XpYpO, 17p7 and 21qseq1).

Statistical methods

Statistical analysis was carried out using Prism 6.0 (Graphpad Software Inc., La Jolla, USA) and SAS version 9.3 software (SAS Institute, Marlow, UK). Univariate comparisons for overall survival (OS) were conducted with the log-rank test and displayed as Kaplan Meier curves. Analyses of time to event outcomes with respect to continuous variables or those with less than two categories, together with multivariable analyses, were performed using a Cox proportional hazard model with forward selection. $P<0.05$ was considered significant.

Results

Telomere length and fusion analysis

We evaluated the telomere length distributions in 200 CLL patients using single telomere length analysis (STELA) at the XpYp telomere (Fig. 1A). STELA allows complete resolution of telomere lengths at specific chromosome ends (Baird*, et al* 2003, Capper*, et al* 2007). We analysed the XpYp telomere because our previous data indicate that it is representative of the genome-wide telomere length (Baird*, et al* 2006, Baird*, et al* 2003) and that telomerase-expressing cells can homogenise telomere lengths at different chromosome ends (Britt-Compton*, et al* 2009, Lin*, et al* 2010). We previously showed that telomere end-end fusion events can be detected in CLL patients with short telomeres (Lin*, et al* 2010), so we systematically assayed for telomere fusions starting with the CLL samples with the shortest telomere length distributions. We only considered a fusion event to be *bone fide* when it could be fully characterized by direct DNA sequence analysis (Fig. 1B, 1C and Fig. S1). Telomere fusions were detectable in samples derived from all Binet stages, suggesting that they are not merely a characteristic of advanced stage disease (Fig. 1D; fusions marked in red). Furthermore, the radical telomere shortening seen in this study was completely distinct from natural telomere erosion observed as a function of age. To reinforce this point, we found no association between telomere length and patient age in our study (r^2 = 0·004). Fusion events were not detected in samples with a mean XpYp telomere length of >3.81 kb and so this upper threshold of telomere dysfunction allowed us to define, for the first time, the full extent of the 'fusogenic' range in a human cancer population. The mean telomere length of the patient subset in which we detected fusions was 2.26 kb (Fig. 1E) and the fusogenic range was (0.44 kb – 3.81 kb). We next assessed the impact of the fusogenic mean as prognostic marker in our cohort and compared its performance with established clinical and laboratory prognostic markers.

Telomere dysfunction improves prognostic resolution in terms of TTFT and PFS

In keeping with previous studies (Damle*, et al* 2004, Roos*, et al* 2008, Rossi*, et al* 2009), categorisation by mean telomere length (3.9 kb) was prognostic in our discovery cohort for both time to first treatment (TTFT) and progression-free survival (PFS) (Fig. 2A and 2B respectively). However, categorisation of the samples above and below the telomere fusogenic mean significantly improved the prognostic discrimination of telomere length measurement for TTFT and PFS (Fig. 2C and 2D) and segregation of the cohort based on the upper limit of fusions (≤/>3.81 kb) also enhanced the prognostic discrimination of mean telomere length measurement (Fig. 2E and 2F).

Telomere dysfunction is strongly predictive of overall survival even in stage A patients

Patients with telomeres below the fusogenic mean (≤2.26 kb) had significantly shorter overall survival (Fig. 3A) when compared to patients above the fusogenic mean (HR = 13.2 (11.6-106.4); P<0.0001). Furthermore, this was also true for patients with early stage disease (HR = 19.3 (17.8-802.5); P<0.0001, Fig. 3B). These findings confirm that telomere erosion and dysfunction can precede clinical disease progression and importantly they allow the accurate stratification of Binet stage A patients into those with indolent disease course and those with a consistently poor prognosis.

Validation of telomere dysfunction in an independent cohort of CLL patients

Given the striking findings from our discovery cohort, we set out to validate our results in an independent cohort of CLL patients derived from a different centre. We performed STELA on an additional 121 unselected CLL samples in a blinded fashion and subsequently analysed the impact of the fusogenic mean on survival for the whole cohort and the stage A patients.

Figures 3C and 3D show that the prognostic impact of telomere dysfunction was clearly evident in the total cohort (P<0.0001; HR = 7.4 (6.9-96.3)) and the stage A cohort (P<0.0001; HR = 8.9 (7.0-320.7)). These findings support the results from the discovery cohort and confirm the prognostic potential of telomere dysfunction in CLL. Furthermore, univariate analysis of the combined discovery and validation cohorts (n = 321) revealed that the fusogenic mean was associated with markedly increased hazard ratios signifying improved prognostic power for PFS and OS than all of the established clinical and laboratory markers evaluated including *IGHV* mutation status, CD38 expression, ZAP-70 expression, fluorescence *in situ* hybridisation cytogenetics and beta-2 microglobulin. These data are summarised in Table 1.

Recursive partitioning identifies the 2.26 kb threshold as most prognostic for survival

Although we had experimentally determined the telomere length threshold for telomere dysfunction in CLL, we wanted to establish if it represented the optimal telomere length cut-off for predicting survival in our cohort. We therefore performed recursive partitioning of the dataset and revealed that telomere length cut-offs within our defined fusogenic range (≤3.81 kb) were highly prognostic when compared to the established prognostic markers, there was a consistent increase in hazard ratio at 2.26 kb demonstrating that this telomere length was the most prognostic threshold in the discovery and validation cohorts (Fig. 3E and 3F respectively).

Telomere dysfunction enhances the prognostic impact of established prognostic markers

Kaplan Meier curves showing the performance of the established prognostic markers in the combined discovery and validation CLL cohort are given in Fig. S2. These markers provided

similar prognostic power to that described in previous studies (Damle*, et al* 1999, Dohner*, et al* 1999, Hamblin*, et al* 1999, Rassenti*, et al* 2008) but all of the markers were considerably less prognostic as evidenced by lower hazard ratios than the telomere fusogenic mean. We therefore assessed whether the fusogenic mean could provide additional prognostic information in the context of established good and bad prognostic subsets. In every case, the telomere fusogenic mean contributed the dominant effect. For example, patients with telomeres below the fusogenic mean showed inferior prognosis regardless of their *IGHV* mutation status. Similarly, patients with telomeres above the fusogenic mean showed superior prognosis regardless of their *IGHV* mutation status. This effect was also observed in patients with 11q- or 17p- cytogenetic lesions i.e. the adverse clinical impact of these abnormalities was only evident in the patients with short telomeres (Fig. 4).

Telomere length is the dominant co-variable in multivariate analysis

In order to test the independent prognostic value for telomere dysfunction in CLL prognosis, we next performed multivariate analysis on the combined CLL dataset using Cox proportional hazards regression with forward selection. The variables included in the model were age at diagnosis, Binet stage at diagnosis, telomere fusogenic mean, *IGHV* mutation status, CD38 expression, ZAP70 expression, cytogenetics. The telomere fusogenic mean was associated with the highest hazard of progression and death independent of all other established biomarkers. Table 2 shows the co-variables included in the model in order of importance. When the telomere fusogenic mean was entered in the model, only *IGHV* mutation status and Binet stage retained independent significance for PFS and only CD38 expression retained independent significance for OS in the model; no other parameters met the $P<0.05$ level for inclusion once the fusogenic mean was entered into the model.

Discussion

We have recently provided the first direct demonstration that human tumour cells can develop telomere dysfunction and undergo a 'telomere crisis' that drives genomic instability and clonal evolution (Lin*, et al* 2010). Given this finding, we set out to establish the prognostic significance of telomere dysfunction in a large unselected cohort of CLL patients (n = 200). As a first step, we identified the upper limit of detection of telomere fusion events allowing us to accurately define, for the first time, the fusogenic range in a primary human malignancy. We then used the mean of the fusogenic range (2.26 kb) to categorise the patient cohort. This new definition of telomere dysfunction transformed the prognostic power of telomere length analysis into the most prognostic parameter ever described in CLL, a finding that was subsequently confirmed in an independent validation cohort of 121 CLL patients. Recursive partitioning of our data independently confirmed that 2.26 kb was the most prognostic threshold in both the discovery and validation cohorts providing further evidence that it represents the biologically important threshold of telomere stability in CLL.

The molecular basis of the extensive telomeric erosion observed in some CLL patients remains largely unexplained. However, given that it can precede disease progression it must represent an early event in the pathology of this disease at least in some patients. A number of recent publications have highlighted somatic mutations in *POT1*, (Ramsay*, et al* 2013, Robles-Espinoza*, et al* 2014) a protein critical for telomere capping, as driver mutations in CLL and malignant melanoma offering a mechanistic rationale for telomeric fusion events. However, *POT1* mutations appear to be exclusively associated with unmutated *IGHV* genes in CLL (Ramsay*, et al* 2013). Therefore they cannot provide a neat explanation for the critically short telomeres observed in this study as only 49% of cases with telomeres within the fusogenic range had unmutated *IGHV* genes.

A number of previous studies using QPCR, TRF or FISH-based techniques have shown that mean telomere length can provide useful prognostic information in CLL but only in combination with other established markers (Ricca*, et al* 2007, Roos*, et al* 2008, Rossi*, et al* 2009). As a consequence telomere length assessment has not been adopted as a routine test in the clinical management of CLL. However, by using high-resolution STELA analysis and applying a telomere length threshold based on telomere dysfunction, we have identified a biomarker of clinical outcome in CLL that provides unprecedented prognostic resolution. It is worthy of note that as STELA is able to accurately measure telomere lengths within this critical fuosgenic range it provides clinical prognostic information beyond the ability of QPCR, TRF or FISH-based techniques (Aubert*, et al* 2012, Baird 2005). Thus, our study transforms the utility of telomere length assessment in CLL and allowed accurate prospective stratification of Binet stage A patients into those with indolent disease (91% survival at 10 years) and those with poor prognosis (13% survival at 10 years). The power of telomere dysfunction to accurately predict clinical outcome in CLL was further reinforced when we performed multivariate analysis on the combined cohort. Telomere dysfunction was the dominant co-variable in Cox proportional hazards forward selection modelling. Although STELA is a multi-step precision assay, its component parts utilise standard molecular biology approaches (PCR and Southern blotting). As such it is no more complex than *IGHV* mutation analysis to perform and is technically much less difficult to perform and interpret than next generation sequencing for example. Given the prognostic power of the assay demonstrated here there is no technical reason why it should not be amenable to widespread implementation in clinical practice.

In conclusion our data strongly support the concept that telomere dysfunction is a critical determinant of clinical outcome in CLL. Our definition of telomere dysfunction provides reliable prognostic information and facilitates, for the first time, confident prediction of clinical outcome for individual patients. This will be of particular utility for the majority of patients who initially present with early stage disease. As such our data provides compelling evidence for the use of high-resolution telomere length analysis coupled with a definition of telomere dysfunction in the prognostic assessment of individual CLL patients.

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Authorship contribution

TTL carried out the experimental work, analysed the data and edited the manuscript; KN carried out the experimental work, analysed the data and edited the manuscript; NHH carried out the experimental work, analysed the data; GP provided clinical samples and data; JMA

provided clinical samples and data; DJA provided clinical samples and data; JB provided clinical samples and data; LC provided clinical samples and data; RH provided expert statistical analysis and edited the manuscript; JWG contributed to the experimental work; REJ contributed to the experimental work; BB-C contributed to the experimental work; CF provided clinical samples and data and edited the manuscript; DMB and CP jointly conceived and supervised the study, analysed the data and wrote the manuscript. All authors contributed to the final version of the manuscript.

Conflict of interest disclosure

TTL declares no conflict of interests; KN declares no conflict of interests; NHH declares no conflict of interests; GP declares no conflict of interests; JMA declares no conflict of interests; DJA declares no conflict of interests; JB declares no conflict of interests; LC declares no conflict of interests; RH declares no conflict of interests; JWG declares no conflict of interests; REJ declares no conflict of interests; BB-C declares no conflict of interests; CF declares no conflict of interests other than co-authorship of a patent application based on some of this work; DMB declares no conflict of interests other than co-authorship of a patent application based on some of this work and CP declares no conflict of interests other than co-authorship of a patent application based on some of this work.

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Table 1. Univariate comparison of prognostic factors in the combined discovery and validation cohorts (n = 321) in terms of overall survival.

Parameter	Progression-free survival			Overall survival		
	Median (years)	**HR (95%CI)**	**P-value**	**Median (years)**	**HR (95%CI)**	**P-value**
Fusogenic mean		**5.6** (4.9-46.6)	<0.0001		**12.8** (10.0-103.8)	<0.0001
≤2.26 kb	2.0			7.2		
>2.26 kb	not reached			not reached		
***IGHV* status**		3.8 (2.6-7.9)	<0.0001		2.9 (1.9-7.0)	<0.0001
≧98%	2.9			10.0 years		
<98%	not reached			not reached		
CD38		2.4 (1.5-4.5)	0.0006		2.3 (1.4 -5.1)	0.003
≥20%	3.0			not reached		
<20%	not reached			not reached		
ZAP-70		2.1 (1.3-2.8)	0.07		1.7 (0.95-3.2)	0.07
≥20%	6.0			not reached		
<20%	not reached			not reached		
β2-M		3.1 (1.6-6.2)	0.001		1.6 (0.69-3.8)	0.28
≥4 mg/l	3.0			not reached		
<4 mg/l	not reached			not reached		
Genetics		4.7 (3.1-14.9)	<0.0001		3.8 (2.8-23.4)	0.0001
$11q^-$ / $17p^-$	2.3			6.9		
N / O	not reached			not reached		

Fusogenic mean: The mean telomere length of the samples in which fusion events were detected
IGHV status: <98% sequence homology with the closest germline sequence (mutated); ≥98% sequence homology with the closest germline sequence (unmutated)
β2-M: beta-2 microglobulin;
$11q^-$ and $17p^-$: any FISH or karyotypic abnormality of 11q or 17p
N: No detectable cytogenetic aberration by FISH; O: Other cytogenetic abnormality (excluding $11q^-$ or $17p^-$)
HR = Hazard ratio; CI, confidence interval
95% CI = 95% confidence interval

Table 2. Multivariate analysis of the combined discovery and validation CLL cohorts using Cox proportional hazards regression with forward selection

Summary of forward selection in order of inclusion in the model							
Progression-free survival				**Overall survival**			
Effect	**HR**	**95% CI**	**p-value**	**Effect**	**HR**	**95% CI**	**p-value**
Fusogenic mean	5.11	2.39-7.07	<0.0001	Fusogenic mean	12.86	4.9-33.5	<0.0001
IGHV status	1.21	1.1-1.32	0.001	CD38 expression	2.67	1.12-6.37	0.026
Binet stage	1.52	1.09-2.12	0.012				

HR = Hazard ratio
95% CI = 95% confidence interval
Variables considered in the model were: age at diagnosis, Binet stage at diagnosis, telomere fusogenic mean, *IGHV* mutation status, CD38 expression, ZAP70 expression, cytogenetics.

Figure Legends

Figure 1. Defining telomeric parameters for prognosis in CLL. (A) shows an example of STELA at the XpYp telomere in 12 CLL patients in which fusion was, or was not detected as indicated above. Mean and standard deviation are displayed below and the means highlighted in red on the gel image. (B) examples of fusion analysis in 4 CLL patients. (C) examples of the DNA sequence of the fusion events highlighted on the fusion gel above. Arrows indicate the fusion junction, together with the participating telomere and the deletion from the start of the respective telomeres. Homology between the participating telomeres is underlined. (D) mean XpYp telomere length data plotted as a function of Binet staging in the discovery cohort. Black markers indicate those that were not tested for fusion, blue those that were negative and red those that were positive for fusion events. (E) telomere length data from the

discovery cohort, together with those that were positive for fusion events. The mean XpYp telomere length of the samples in which fusion was detected was 2.26 kb.

Figure 2. Telomere dysfunction predicts time to first treatment and progression-free survival in CLL. (A) and (B) In keeping with previous studies, mean telomere length (as measured by STELA) is prognostic for TTFT and PFS respectively. Segregation of the discovery cohort into subsets defined by the fusogenic mean (≤2.26 kb) significantly enhanced the prognostic resolution for (C) TTFT and (D) PFS. Furthermore, the upper limit of fusions (≤3.81 kb) also improved prognostic discrimination for (E) TTFT and (F) PFS) but to a less extent than the fusogenic mean.

Figure 3. Telomere length as defined by fusion is highly prognostic for overall survival in CLL. (A) and (B) Kaplan Meier curves for overall survival in the discovery cohort of 200 CLL patients and the stage A subset (n = 144) respectively. (C) and (D) show Kaplan Meier curves for overall survival in the validation cohort of 121 CLL patients and the stage A subset (n = 92). P values, Hazard Ratio (HR) and 95% confidence intervals are indicated on the plots together with numbers at risk in each arm. (E) recursive partitioning of mean telomere length in our initial dataset and plots the hazard ratios for overall survival for each threshold for the entire cohort and the stage A patient subset. (F) recursive partitioning of the validation cohort and the stage A subset. In every case 2.26 kb provided the optimal discrimination.

Figure 4. Telomere dysfunction effectively negates the impact of established prognostic markers. Survival curves for concordant and discordant datasets based on telomere dysfunction in combination with (A) *IGHV* status, (B) CD38 status, (C) FISH cytogenetics and (D) ZAP70 status in the combined discovery and validation cohort (n = 321). The

prognostic effect of telomere dysfunction dominates the analysis to such an extent that it effectively removes the negative impact of established poor prognostic markers.

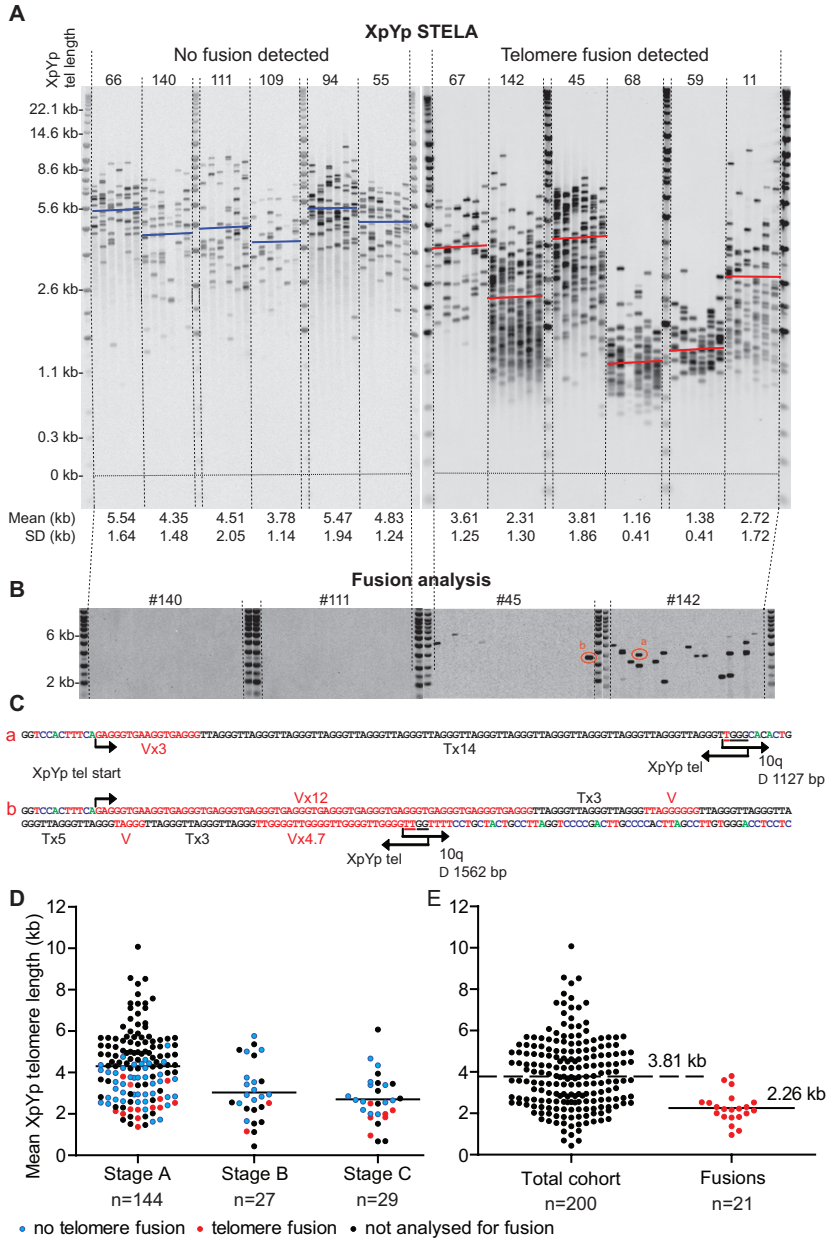
A
XpYp STELA
No fusion detected
Telomere fusion detected
XpYp tel length
66 140 111 109 94 55 67 142 45 68 59 11
22.1 kb
14.6 kb
8.6 kb
5.6 kb
2.6 kb
1.1 kb
0.3 kb
0 kb
Mean (kb) 5.54 4.35 4.51 3.78 5.47 4.83 3.61 2.31 3.81 1.16 1.38 2.72
SD (kb) 1.64 1.48 2.05 1.14 1.94 1.24 1.25 1.30 1.86 0.41 0.41 1.72
B
Fusion analysis
#140
#111
#45
#142
6 kb
2 kb
C
a
Vx3
Tx14
XpYp tel start
XpYp tel
10q
D 1127 bp
b
Vx12
Tx3
V
Tx5
V
Tx3
Vx4.7
XpYp tel
10q
D 1562 bp
D
Mean XpYp telomere length (kb)
Stage A
n=144
Stage B
n=27
Stage C
n=29
E
3.81 kb
2.26 kb
Total cohort
n=200
Fusions
n=21
no telomere fusion
telomere fusion
not analysed for fusion

Fig. 1

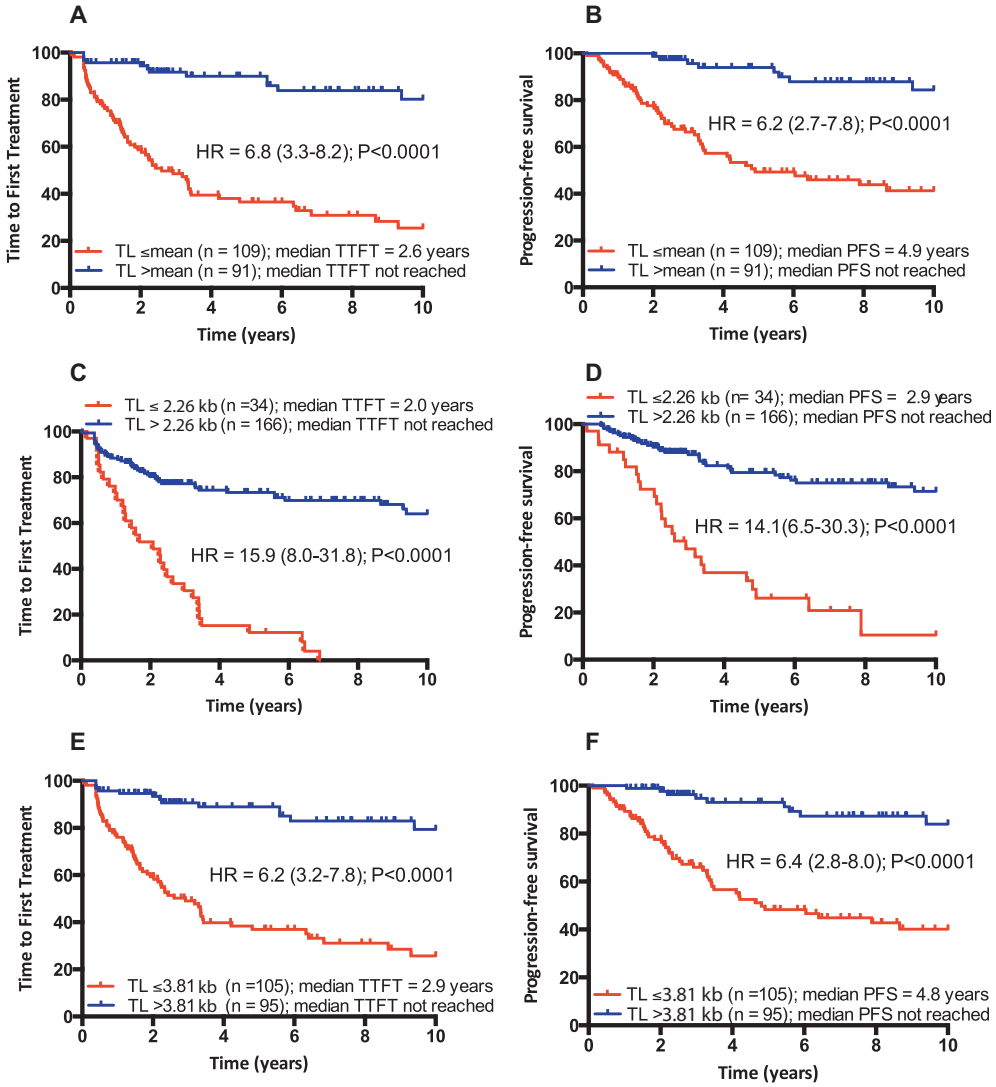
A
Time to First Treatment
HR = 6.8 (3.3-8.2); P<0.0001
TL ≤mean (n = 109); median TTFT = 2.6 years
TL >mean (n = 91); median TTFT not reached
Time (years)
B
Progression-free survival
HR = 6.2 (2.7-7.8); P<0.0001
TL ≤mean (n = 109); median PFS = 4.9 years
TL >mean (n = 91); median PFS not reached
Time (years)
C
TL ≤ 2.26 kb (n =34); median TTFT = 2.0 years
TL > 2.26 kb (n = 166); median TTFT not reached
Time to First Treatment
HR = 15.9 (8.0-31.8); P<0.0001
Time (years)
D
TL ≤2.26 kb (n= 34); median PFS = 2.9 years
TL >2.26 kb (n = 166); median PFS not reached
Progression-free survival
HR = 14.1(6.5-30.3); P<0.0001
Time (years)
E
Time to First Treatment
HR = 6.2 (3.2-7.8); P<0.0001
TL ≤3.81 kb (n =105); median TTFT = 2.9 years
TL >3.81 kb (n = 95); median TTFT not reached
Time (years)
F
Progression-free survival
HR = 6.4 (2.8-8.0); P<0.0001
TL ≤3.81 kb (n =105); median PFS = 4.8 years
TL >3.81 kb (n = 95); median PFS not reached
Time (years)

Fig. 2

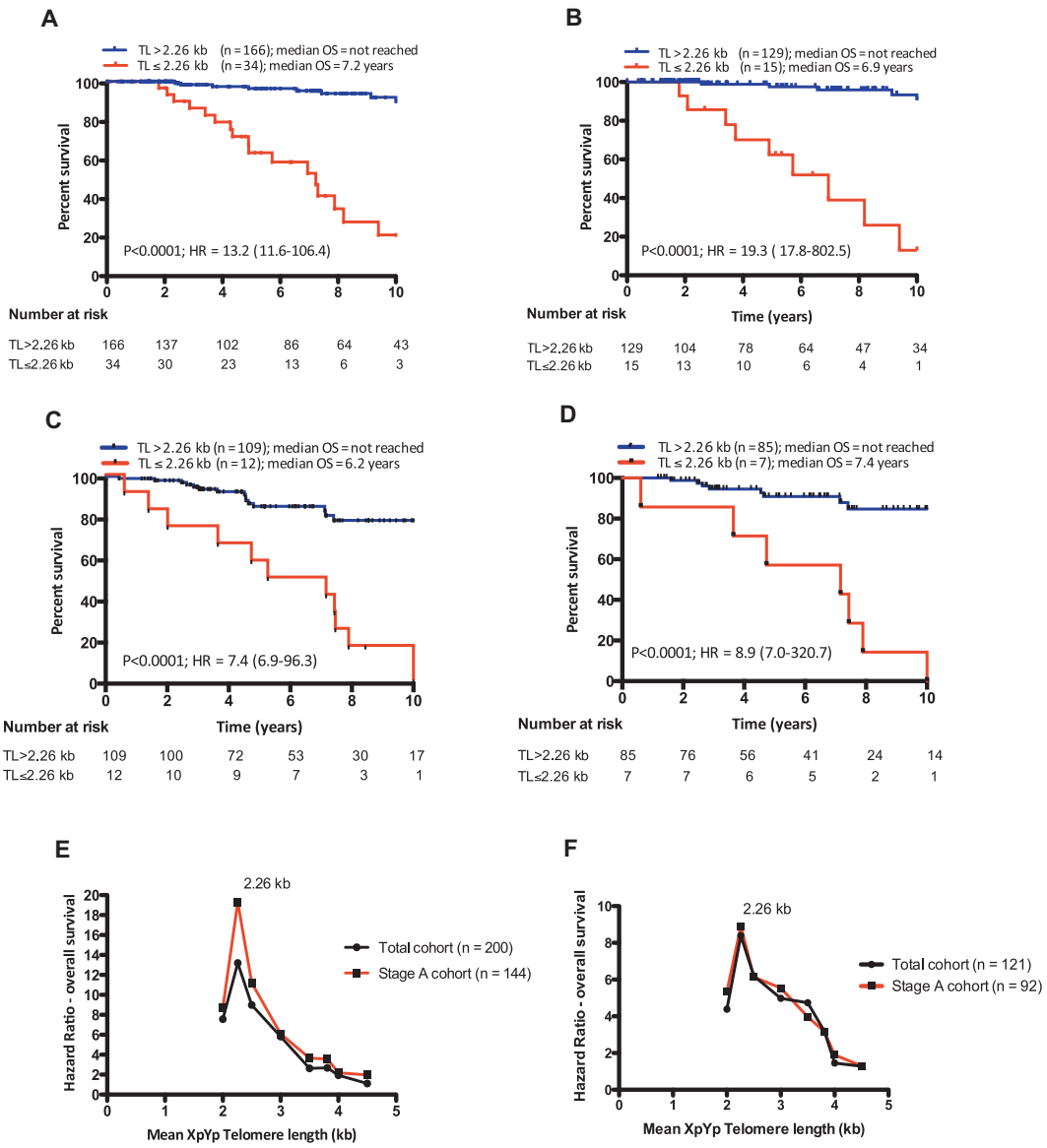
A
TL > 2.26 kb (n = 166); median OS = not reached
TL ≤ 2.26 kb (n = 34); median OS = 7.2 years
Percent survival
P<0.0001; HR = 13.2 (11.6-106.4)
Number at risk
TL>2.26 kb 166 137 102 86 64 43
TL≤2.26 kb 34 30 23 13 6 3
B
TL > 2.26 kb (n = 129); median OS = not reached
TL ≤ 2.26 kb (n = 15); median OS = 6.9 years
Percent survival
P<0.0001; HR = 19.3 (17.8-802.5)
Number at risk
Time (years)
TL>2.26 kb 129 104 78 64 47 34
TL≤2.26 kb 15 13 10 6 4 1
C
TL > 2.26 kb (n = 109); median OS = not reached
TL ≤ 2.26 kb (n = 12); median OS = 6.2 years
Percent survival
P<0.0001; HR = 7.4 (6.9-96.3)
Number at risk
Time (years)
TL>2.26 kb 109 100 72 53 30 17
TL≤2.26 kb 12 10 9 7 3 1
D
TL > 2.26 kb (n = 85); median OS = not reached
TL ≤ 2.26 kb (n = 7); median OS = 7.4 years
Percent survival
P<0.0001; HR = 8.9 (7.0-320.7)
Number at risk
Time (years)
TL>2.26 kb 85 76 56 41 24 14
TL≤2.26 kb 7 7 6 5 2 1
E
2.26 kb
Hazard Ratio - overall survival
Total cohort (n = 200)
Stage A cohort (n = 144)
Mean XpYp Telomere length (kb)
F
2.26 kb
Hazard Ratio - overall survival
Total cohort (n = 121)
Stage A cohort (n = 92)
Mean XpYp Telomere length (kb)

Fig. 3

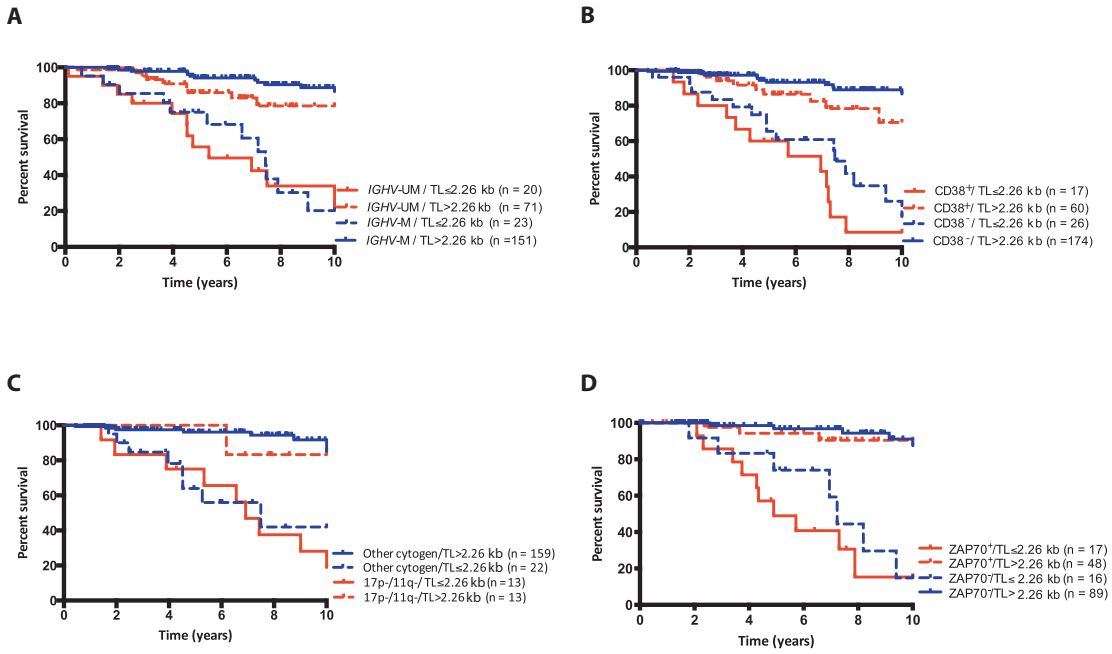
A
Percent survival
Time (years)
IGHV-UM / TL≤2.26 kb (n = 20)
IGHV-UM / TL>2.26 kb (n = 71)
IGHV-M / TL≤2.26 kb (n = 23)
IGHV-M / TL>2.26 kb (n =151)
B
Percent survival
Time (years)
CD38+/ TL≤2.26 kb (n = 17)
CD38+/ TL>2.26 kb (n = 60)
CD38-/ TL≤2.26 kb (n = 26)
CD38-/ TL>2.26 kb (n =174)
C
Percent survival
Time (years)
Other cytogen/TL>2.26 kb (n = 159)
Other cytogen/TL≤2.26 kb (n = 22)
17p-/11q-/ TL≤2.26 kb (n = 13)
17p-/11q-/TL>2.26 kb (n = 13)
D
Percent survival
Time (years)
ZAP70+/TL≤2.26 kb (n = 17)
ZAP70+/TL>2.26 kb (n = 48)
ZAP70-/TL≤ 2.26 kb (n = 16)
ZAP70-/TL> 2.26 kb (n = 89)

Fig. 4