Telomere length is an independent prognostic marker in MDS but not in de novo AML

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Telomere length is an independent prognostic marker in MDS but not in de novo AML

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Summary

Telomere dysfunction is implicated in the generation of large-scale genomic rearrangements which drives progression to malignancy. In this study we used high-resolution single telomere length analysis (STELA) to examine the potential role of telomere dysfunction in 80 Myelodysplasia (MDS) and 95 de novo Acute Myeloid Leukaemia (AML) patients. Despite the MDS cohort being older they had significantly longer telomeres than the AML cohort (P < .0001) where telomere length was also significantly shorter in younger AML patients (age <60) (P = .02) and in FLT3 ITD mutated AML patients (P = .03). Using a previously determined telomere length threshold for telomere dysfunction (3.81kb) did not provide prognostic resolution in AML (HR = 0.68, P = .2). In contrast, the same length threshold was highly prognostic for overall survival in the MDS cohort (HR = 5.0, P < .0001). Furthermore, this telomere length threshold was an independent parameter in multivariate analysis when adjusted for age, gender, cytogenetic risk group, number of cytopenias and IPSS score (HR = 2.27, P < .0001). Therefore, telomere length should be assessed in a larger prospective study to confirm its prognostic role in MDS with a view to integrating this variable into a revised IPSS.
Introduction

The Myelodysplastic Syndromes (MDS) are a heterogeneous group of clonal haematopoietic disorders with varying survival and propensity to develop secondary acute myeloid leukaemia (sAML). In 1997 the International Prognostic Scoring System (IPSS) was based on the bone marrow blast count, 3 distinct cytogenetics risk groups and the number of cytopenias dividing patients into four IPSS subgroups (Greenberg, et al 1997). The IPSS – R (Revised) is an updated refinement of the IPSS which identifies five cytogenetic, three cytopenic and four blast count risk categories which combine into five overall prognostic subgroups (Greenberg, et al 2012, Vardiman, et al 2009). Many MDS patients have a normal karyotype but in recent years a very large number (up to 660) of molecular genetic defects have been identified encoding genes for cellular proteins including transcription factors e.g. RUNX1, epigenetic regulators and chromatin remodelling factors e.g. TET2, DNMT3A, IDH1/2, pre-RNA splicing factors e.g. SF3B1, receptor tyrosine kinase/ signalling molecules e.g. NRAS, JAK2, NPM1,FLT3 and check point regulator P53 some of which impact significantly on prognosis (Tothova, et al 2013, Walter, et al 2012). A recent MDS study showed that some of these somatic mutations were an independent prognostic marker compared to the IPSS (Bejar, et al 2011).

Recent studies have shown that the same genomic mutations seen in MDS/AML patients overlap with those identifiable in the normal adult population with increasing frequency with age (Genovese, et al 2014, Jaiswal, et al 2014, McKerrell, et al 2015, Xie, et al 2014). What drives these clones to further genomic instability and the development of MDS/AML is currently poorly understood. Telomeres are repetitive DNA sequences at the ends of chromosomes that shorten with each cell division. Critical loss of telomere length leads to chromosome end-end fusion and genomic instability resulting in large scale re-
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arrangements such as non-reciprocal translocations which are the hallmark of many tumour
types including MDS, sAML and *de novo* AML (Jones, *et al* 2012). With ongoing cell division
during life, the telomeres in normal individuals erode as a function of age at a rate of
approximately 26 bp/year (Daniali, *et al* 2013). Several studies have suggested that MDS is
associated with shorter telomeres leading to genomic instability and progression to sAML
Young 2010). Recently, using a modified Q-FISH based method, Gadji *et al* proposed that
telomere dysfunction underpins the chromosomal changes associated with MDS
progression to AML and *de novo* AML (Gadji, *et al* 2012). Whilst in a mouse model it was
shown that telomere dysfunction induced the same types of DNA damage that drives
classical MDS phenotypes including *SF3B1* and *DNMT3A* resulting in differentiation changes
in myeloid precursors (Colla, *et al* 2015).

We previously developed a high-resolution technique to determine telomere length, Single
Telomere Length Analysis (STELA), which is unique in its ability to detect telomere lengths
from single chromosomes within the length ranges at which telomere fusions can occur (Lin,
*et al* 2010, Lin, *et al* 2014). Using STELA we have shown that some chronic lymphocytic
leukaemia (CLL) patients display extreme telomere shortening and fusion consistent with
the onset of a telomere-driven crisis that can drive the formation of large-scale genome
rearrangements (Lin, *et al* 2010). Telomeres in these ranges cannot be readily detected with
the other methodologies such as QPCR, Southern blot or Q-FISH previously used in
*et al* 2010). We showed that in CLL patients, telomere fusions only occurred when telomere
length was ≤3.81 kb and importantly we show that telomere erosion to within these length
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ranges precedes clinical progression. Indeed high-resolution telomere length analysis using
STELA, together with the stratification of patients based on the telomere length thresholds
at which fusion occurs, provided an independent high-resolution marker of prognosis even

In this study we used STELA to assess if telomere erosion is an important pathogenic
mechanism driving prognosis MDS and AML.

Methods

Patients, samples and cell separation

This study was undertaken at the University Hospital of Wales (UHW), Cardiff. All of the
unselected diagnostic patient bone marrow samples: 80 MDS which consisted of 37 samples
from Dundee (archived from 1997 – 2005) and 43 from Cardiff (archived between 1985 and
2008) including 7 with a proven and documented history of MDS prior to progression to
AML (blasts >20%), and 95 de novo AML from Cardiff (archived between 2003 and 2012)
were obtained following written informed consent (Table 1). We deliberately chose to use
stored but well annotated archival samples so as to be able to assess the potential impact of
telomere length on survival in all patient groups but especially low risk MDS patients. All
MDS patients were treated with best supportive care with none receiving azacitidine prior
to this analysis. All morphological, immunophenotypic, cytogenetic and molecular data
were collected from Cardiff and Dundee Haematology Departments for their respective
patients but all telomere length analyses on the bone marrow mononuclear cells were
undertaken within the Division of Cancer and Genetics, Cardiff University. Bone marrow
mononuclear cells were collected in ethylenediaminetetraacetic acid and isolated by
density centrifugation using Ficoll-Hypaque (Invitrogen) which resulted in <3%
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lymphocytes contamination. Cells were stored at −20°C as dry pellets before DNA extraction.

DNA extraction, single telomere length analysis and telomerase assays.

Telomere length was determined using XpYp STELA as previously described (Lin, et al 2010, Roger, et al 2013). Briefly, DNA was extracted using proteinase K, RNase A, phenol/chloroform protocols and quantified by Hoechst 33258 fluorometry (Bio-Rad) before dilution to 10 ng/μL in 10mM Tris-HCl, pH 7.5. A total of 10 ng of DNA was further diluted to 250 pg/μL in a volume of 40 μL containing 1μM Telorette2 linker and 1mM Tris-HCl, pH 7.5. Multiple polymerase chain reactions (PCRs; typically 6 reactions per sample) were carried out for each test DNA in 10-μL volumes 250 pg of DNA, 0.5μM of the telomere-adjacent and Teltail primers, 75mM Tris-HCl, pH 8.8, 20mM (NH4)2SO4, 0.01% Tween-20, 1.5mM MgCl2, and 0.5 U of a 10:1 mixture of Taq (ABGene) and Pwo polymerase (Roche Molecular Biochemicals). The reactions were cycled with an MJ PTC-225 thermocycler (MJ Research). The DNA fragments were resolved by 0.5% Tris acetate ethylenediaminetetraacetic acid agarose gel electrophoresis, and detected by Southern blot hybridization with random-primed α-33P–labelled (GE Healthcare) TTAGGG repeat probe together with probes to detect the 1-kb (Stratagene) and 2.5-kb (Bio-Rad) molecular weight markers. The hybridized fragments were detected by phosphorimaging with a Molecular Dynamics Storm 860 phosphorimager (GE Healthcare). The molecular weights of the DNA fragments were calculated using the Phoretix 1D quantifier (Nonlinear Dynamics). Telomerase assays were undertaken using the TRAPeze XL Telomerase detection kit (Chemicon International, Billerica, MA) as previously described (Lin, et al 2010, Roger, et al 2013).
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Statistical methods

Statistical analysis was undertaken by the Haematology Clinical Trials Unit, Cardiff University using SAS version 9.4 and GraphPad Prism 6. Spearman’s correlation was used for correlations between baseline values; Mann-Whitney and Wilcoxon matched-paired nonparametric tests were used for comparisons between groups. Mean telomere length was assessed between diagnoses using the Wilcoxon rank sum test. Paired data were compared using the Wilcoxon signed rank test. In the MDS cohort patients were assessed using the 1997 IPSS criteria for the 3 cytogenetic risk groups, blast count, number of cytopenias (Hb <100g/l, neutrophils <1.5x10⁹/l and platelets <100x10⁹/l), IPSS score, age and sex whereas the AML cohort was assessed for cytogenetic risk group and FLT3 and NPM mutations (Falini, et al 2005, Kottaridis, et al 2001, Townsley, et al 2014). Survival was assessed using the Kaplan-Meier method, and compared using Cox proportional hazards regression, with model building carried out using forward selection with significance set at P = .05.

Results

Telomere length MDS and AML patients.

An MDS patient’s bone marrow will typically display a variable range of immature and more differentiated cells which may or may not be derived from the malignant stem cells. We therefore compared the telomere length of the first 20 individual MDS patients CD34+ and CD34- bone marrow cells, but found no significant difference in telomere length between the two fractions (P = .08; Supplementary Figure 1A). We therefore proceeded without cell
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160 selection in subsequent MDS patient bone marrow sample analyses and simply used bone
161 marrow mononuclear cell pellets.

162 Despite the fact that the MDS patients (median age 68 years, range 21-86) were older than
163 AML patients (median age 56 years, range 17-80) telomere length was significantly longer in
164 the MDS cohort compared to the AML cohort (P < .0001; Figure 1A). Although MDS samples
165 showed a modest reduction in telomere length with increasing age at diagnosis, there was
166 no significant correlation ($\rho^2 = -.0212, P = .2$; Figure 1B) whereas in the AML samples a
167 positive correlation was observed ($\rho^2 = .0890, P = .003$; Figure 1C). This finding is in contrast
168 to what occurs during normal ageing; samples derived from older AML patients (age >60)
169 had significantly longer telomeres than younger AML patients (P = .02; Figure 1D). Indeed,
170 22/26 (84.6%) of AML patients age <50 years had telomere lengths within the range at
171 which they can become dysfunctional or ‘fusogenic’ ($\leq 3.81$ kb), which we previously
described in CLL (Lin, et al 2014).

173

174 Telomere length, blast count, cytogenetics, cytopenias and IPSS.

175 We next analysed the MDS cohort to assess any possible correlations between telomere
176 length and age, gender, blast count, number of cytopenias, cytogenetic risk group and IPSS
177 sub-group. Shorter telomere length was associated with male gender (P = .01;
178 Supplementary Figure 1B) and increased number of cytopenias (P = .003; Figure 2A). None
179 of the other parameters were significantly associated with telomere length. Consistent with
180 previous reports we found a significant association between the number of cytopenias and
181 overall survival (P < .0001; Supplementary Figure 2A). Furthermore, patients with high risk
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cytogenetic abnormalities tended to have worse survival although this did not reach statistical significance (P = .12; Supplementary Figure 2B). Interestingly we found no simple association between telomere length and the three IPSS cytogenetic risk groups (P = .6 for trend; Figure 2B), the four IPSS subgroups (\(\rho^2 = .14\) for correlation; Figure 2C) or bone marrow blast percentage (\(\rho^2 = -0.22, P = .0503\) Figure 2D).

In the de novo AML cohort we assessed the correlation between telomere length and gender, age, presenting WBC, performance status, whether primary or secondary AML and NPM1 and FLT3 mutation status. There was no association between NPM1 mutated patients and telomere length (data not shown), but significantly shorter telomeres were found in the FLT3 ITD mutated group when compared to FLT3 wild type AML patients (P = .03). In contrast, there was a trend towards longer telomeres in the FLT3 TKD mutated group compared to ITD mutated AML (P = .12; Figure 2E).

Telomerase activity in MDS and AML patients.
Given the different telomere length characteristics of the MDS and AML cohorts, we investigated whether this may reflect differences in telomerase activity, an enzyme responsible for extending shortened telomeres. Telomerase activity was analysed in a subset of CD34+ AML samples (n = 12) and in purified CD34+ cells from MDS patients (n = 20). Telomerase activity was significantly higher in the AML samples when compared with CD34+ MDS cells (P = .0002; Figure 2F).

Telomere length and survival in MDS and AML.
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We next assessed the impact of telomere length on overall survival in the MDS and AML cohorts. Segregation of the two cohorts according to whether their mean telomere length was above or below the upper limit of telomere dysfunction (3.81 kb) revealed no difference in survival in the AML patient group (HR = 0.68 (0.37-1.9), P = .2; Figure 3A). In contrast, bifurcation of the MDS cohort using this threshold telomere length demonstrated that patients with a median telomere length ≤3.81 kb had significantly worse survival (HR = 5.0 (2.7-10.0), P< .0001; Figure 3B). The impact of telomere length in these two disease settings was confirmed using telomere length quartile analysis. There was no correlation between telomere length and overall survival or relapse-free survival in the AML cohort (P = .5, P = .09; Supplementary Figures 3A and 3B). However, similar quartile analysis in the MDS cohort clearly showed that patients in the lowest two quartiles had worse survival with all patients in the lowest quartile alive at 3 years (Supplementary Figure 3C).

Telomere length is an independent prognostic variable in MDS.

Finally, we performed multivariate analysis in the MDS cohort using a forward selection model that included age, IPSS, gender, cytogenetic risk group, number of cytopenias and telomere length. Short telomere length was identified as the most significant independent marker of overall survival (HR = 2.27 (1.45-3.57), P< .0001). When entering short telomere length into the model the following parameters retained independent prognostic value: high IPSS (HR = 1.2 (0.64-2.27), increased number of cytopenias (HR = 1.60 (1.09-2.35), P = .007), older age (HR per year 1.03 (1.01-1.07), P = .05) and male gender (HR = 2.70 (1.20-6.10), P = .01).
Discussion

We previously showed that a proportion of chronic lymphocytic leukaemia (CLL) patients display extreme telomere erosion and fusion consistent with a telomere-driven crisis. Importantly, this was not simply a function of advanced stage disease but was detected in a subset of early stage patients prior to clinical progression (Lin, et al 2010).

Subsequently we showed that defining the specific telomere length threshold at which telomere fusion occurred was a powerful way to risk-stratify CLL patients even those with early-stage disease (Lin, et al 2014). Previous studies had suggested a possible role for telomere dysfunction in both MDS and AML, so here we investigated the relationship between telomere length, disease progression and clinical outcome in MDS and AML using the high-resolution STELA technique.

We demonstrated that telomere length was highly predictive of disease outcome in MDS. In contrast, we found no evidence that telomere length influenced the survival of de novo AML patients. It should be noted, however, that we demonstrated shorter telomere lengths in the FLT3-ITD mutated group, a group with a well-established poorer prognosis. These results are similar to those of Aalbers et al who also showed shorter telomeres in patients with FLT3-ITD mutations but not NPM1 mutated patients (Aalbers, et al 2013). One possible explanation for the different telomere length characteristics in MDS and AML may be the differential expression of telomerase found in these two conditions. Unlike MDS cells, AML cells showed evidence of upregulated telomerase activity, which could prevent replicative
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Our study showed an association between telomere length and the number of cytopenias in MDS patients. It seems possible that the lack of telomerase activity observed in MDS leads to unhindered cell senescence and/or apoptosis. Also the high proliferative and apoptotic rates seen in MDS bone marrow produces increased ineffective haematopoiesis and more profound cytopenias (Raza, et al 1997a, Raza, et al 1997b). These results are similar to those of Sieglova et al who showed MDS patients with shorter telomeres were more likely to progress to AML (Sieglova, et al 2004). Perhaps surprisingly, there was no association between telomere length and cytogenetic risk group, blast count and IPSS score. This lack of association meant that telomere length was an independent marker of outcome in MDS and in the multivariate forward selection model we employed. Indeed, it was more prognostic than blast count, cytogenetics, number of cytopenias or IPSS in this context.

Several recent studies have shown that clonal haematopoiesis is almost a “normal” part of ageing with recent reports showing 0.8%, 11% and 19.5 % of normal individuals aged <60, >80 and >90 years respectively having demonstrable clonal haematopoiesis – so called age-related clonal haematopoiesis. These include the acquisition of many genetic lesions
Telomere length is prognostic in MDS but not AML


Three of our observations in this study were that the telomere length increases with age in AML patients, that AML patients older than 60 years having significantly long telomeres than those age <60 years and that the AML patient cohort had significantly shorter telomeres than the MDS patient cohort despite being younger. One possible explanation for these data is that older de novo AML patients may have developed secondary AML despite the absence of a documented history of prior MDS or clonal haematopoiesis. This would be in keeping with the recent identification of increased genomic instability in ageing “normal” people with many identical genomic abnormalities seen in elderly AML patients (McKerrell, et al 2015). The acquisition of the various differing leukaemia-associated genomic abnormalities are age dependent with for example the recurrent point mutations affecting spliceosome genes SF3B1 and SRSF2 associated with clonal haematopoiesis in individuals aged 70 years or over, but not in younger people (McKerrell and Vassiliou 2015, Papaemmanuil, et al 2013). An alternative, but not exclusive hypothesis, is that AML in younger patients tends be more progenitor-type AML (Core binding factor, NPM1-mutated, FLT3-ITD-mutated) and more proliferative leading to shorter telomeres. Further studies are required to assess the relationship of shorter telomeres with age-related clonal haematopoiesis and MDS and AML development.

The findings in MDS are in keeping with our previous data in CLL and breast cancer where we demonstrated the utility of our telomere-length threshold in providing powerful independent prognostic information (Lin, et al 2014, Roger, et al 2013). Taken together these data point to a common mechanism that is present in diverse tumour types, by which
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the presence of short dysfunctional telomeres can drive genomic instability and clonal

296 evolution leading to poor clinical outcomes.

297 Our study does have several limitations in that it consists of relatively small cohorts of MDS

298 and AML patients and was deliberately retrospective so as to be able to assess the impact of

299 telomere length on survival especially in low risk MDS patients, all of whom were treated

300 with supportive care only. Finally our observation that telomere length is independently

301 prognostic in MDS indicates that consideration should be given to a much larger prospective

302 study assessing the potential role of telomeres in the prognostication of MDS. This would

303 also facilitate the assessment of telomere length analysis as a potential predictor of

304 response to newer therapies such as azacitidine and perhaps its ultimate incorporation into

305 a revised IPSS.

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308 Performed the research – JW, MHH, BB-C, JWG, REJ, DMB

309 Designed the research study – CF, CP, DMB

310 Contributed essential reagents or tools –ST, MG, DTB, SK

311 Analysed the data – CP, DMB, RKH

312 Wrote the paper – CP, DMB, CF

313 This work was funded by Cancer Research UK and Bloodwise grants 12049 and 13033.
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References


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**Figures and Legends.**

**Table 1.** Demographics of MDS and AML Cohorts

**Figure 1.** (A) Telomere length in the MDS cohort was significantly longer than those of the AML cohort despite being older median age 68 v 56 years (p < .0001). B) In MDS patients there was no correlation between telomere length and age of diagnosis ($\rho^2 = .0212; P = .2$). C) In AML patients there was a positive correlation between telomere length and age of diagnosis ($\rho^2 = .0890; P = .003$). D) Older AML patients (age >60) had significantly longer telomeres than younger patients (P = .02).

**Figure 2.** (A) In the MDS cohort there was a significant association between the telomere length and the number of cytopenia but not with (B) cytogenetic risk group (P = .6 for trend) or (C) IPSS sub-groups ($\rho^2$=.14 for correlation) or (D) blast counts ($\rho^2$=.22 P = .503). E) Patients with a FLT3-ITD showed significantly shorter telomeres than the FLT3-WT group (overall P = .03 and there was a trend towards shorter telomere length in the FLT3-ITD group when compared with the FLT3-TKD group (P = .12)). F) Telomerase activity was significantly higher in AML CD34+ cells compared to MDS CD34+ cells (P = .0002).

**Figure 3.** (A) Using our previously described CLL fusogenic length threshold (TL ≤3.81 kb) there is no difference in survival in AML patients (HR = 1.47 (0.80-2.68), P = 0.2. (B) In contrast, categorization of the MDS cohort above and below the fusogenic length threshold (3.81 kb) demonstrated that MDS patients with short telomeres had an inferior survival (HR = 5.0 (2.7-10.0), P <.0001).

**Supplementary Figure 1.** (A) In 20 MDS patients bone marrow mononuclear cells were sorted but there was no significant difference in telomere length in CD34+ and CD34 – selected sub-populations. (B) In the MDS cohort longer telomere length was associated with female sex (P = .01).
Supplementary Figure 2. (A) In the MDS cohort there was a significant association between the number of cytopenias and overall survival (P < .0001). (B) In the MDS cohort patients with high risk cytogenetic lesions tended to have worse survival although this did not reach statistical significance probably due to the relatively small sample size (P = .12).

Supplementary Figure 3. Quartile analysis of telomere length revealed no significant difference in (A) overall survival and (B) relapse-free survival in the de novo AML cohort. In contrast, (C) MDS patients in the lower two telomere length quartiles showed significantly shorter overall survival.
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Williams et al. Supplementary Figure 1

A

P = .08

XpYp telomere (kb)

CD34+  CD34+

B

P = .01

XpYp telomere (kb)

MALE  FEMALE

Mean (kb)  3.95  4.74
SD (kb)    1.4    1.4
N          50    30

Supplementary Figure 1

177x283mm (600 x 600 DPI)
Williams et al. _Supplementary Figure 2_

**A** MDS Cohort: Overall Survival

- 1 cytopenia
- 2 cytopenias
- 3 cytopenias

At risk:

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**B** MDS Cohort: Overall Survival

- Good risk
- Intermediate risk
- Poor risk

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Supplementary Figure 2

165x244mm (600 x 600 DPI)
Supplementary Figure 3

226x416mm (600 x 600 DPI)