Telomere length is a critical determinant for survival in multiple myeloma

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Summary

Patients with Multiple Myeloma (MM) exhibit variable clinical outcomes, which are incompletely defined by the current prognostication tools. We examined the clinical utility of high-resolution telomere length analysis as a prognostic marker in MM. Stratification of the cohort using a previously determined length threshold for telomere dysfunction revealed that patients with short telomeres had a significant shorter overall survival ($P<0.0001$; HR=3.4). Multivariate modelling using forward selection revealed that the most important prognostic factor was ISS, followed by age and telomere length. Importantly, each ISS prognostic subset could be further risk-stratified according to telomere length, supporting the inclusion of this parameter as a refinement of the ISS.

Despite the introduction of novel therapeutic modalities, patients with multiple myeloma (MM) display a heterogeneous clinical course, with survival ranging from a few months to over 10 years. Therefore, there is a requirement for reliable prognostic and predictive markers in this disease to allow for risk stratification and rational clinical decision-making. The most commonly used prognostic system in MM is the International Staging System (ISS) that is based on serum levels of both β_2-micoglobulin and albumin (Greipp*, et al* 2005). Recently the ISS system has been improved upon by the inclusion of cytogenetic information to take into account the considerable genetic heterogeneity known to occur in this disease and the level of lactate dehydrogenase (Palumbo*, et al* 2015). Hyperdiploidy and the loss of whole chromosome arms is frequently detected in MM, this includes, amongst others, gains of 1q in 30% of cases and the loss of 17p in 7% of cases (Walker*, et al* 2010).

Short dysfunctional telomeres are susceptible to DNA repair activities that can result in chromosomal fusion and the initiation of cycles of anaphase-bridging, breakage and fusion that can drive genomic instability and clonal evolution (Artandi*, et al* 2000, Jones*, et al* 2014, Roger*, et al* 2013). Telomere dysfunction has been documented in numerous haematological malignancies (Jones*, et al* 2012), this is one putative mechanism that may lead to the genetic and clinical heterogeneity observed in MM (Wu*, et al* 2003), and may relate to changes in the 3D telomeric architecture that have been documented in MM cells (Klewes, et al 2013).. Recently we have shown that high-resolution telomere analysis, combined with a functional definition of telomere length, can provide powerful prognostic information in several tumour types, including chronic lymphocytic leukaemia (CLL)(Lin*, et al* 2014), myelodysplasia

(Williams et al. in prep) and breast cancer (Simpson*, et al* 2015). Here we sought to apply these technologies to examine the prognostic utility of telomere length in MM.

Materials and Methods

Patients samples and cell separation

Patient samples were collected at diagnosis, prior to treatment, through treating centres within the Heart of England NHS Foundation Trust and the Newcastle upon Tyne NHS Foundation Trust between 1990 and 2005, with ethical approval from the Newcastle Haematology Biobank (07/H0906/109+5) in accordance with the declaration of Helsinki. Bone marrow samples from five MM patients were fractionated into CD138 + and CD138 - 12 cells using positive selection with CD138+ microbeads and the AutoMACS system (Miltenyi). Cytogenetics was not available on these samples and thus the original ISS staging system was used for this study (Greipp, et al 2005).

DNA extraction and single telomere length analysis

DNA was extracted from sorted cell populations using the Qiagen DNeasy blood and tissue extraction kit, according to the manufacturers instructions. Single telomere length analysis (STELA) at the XpYp telomere was performed as previously described (Baird, et al 2003, Britt-Compton, et al 2006, Capper, et al 2007). A minimum of 6 PCR reactions per sample were carried out for each test DNA and DNA fragments were resolved by agarose gel electrophoresis, and detected by Southern hybridisations and phosphorimaging.

Statistical methods

Statistical analysis was carried out using Prism 6.0 (Graphpad) and SAS version 9.3

software (SAS Institute). Univariate comparisons for overall survival (OS) were conducted with the log-rank test and displayed as Kaplan Meier curves. $P < .05$ was considered significant. 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 and discussion

We used Single Telomere Length Analysis (STELA) of the XpYp telomere to generate telomere length profiles from unsorted bone marrow samples derived from a cohort of 61 patients with MGUS and 134 patients with MM (Figure 1A). STELA is a high-resolution technology capable of detecting telomeres within the length ranges at which telomere fusion is known to occur (Letsolo*, et al* 2010, Lin*, et al* 2014). STELA also provides information on telomere length distributions, which relate to the clonality, replicative history and the cell purity of the tumour cell population analysed. At the individual patient level, it was apparent that the telomeres in both the MGUS and MM bone marrow samples displayed considerable length heterogeneity (Figure 1A), with an overall mean of the SDs obtained from these distributions in the MGUS cohort of 2.23 kb and 2.12 kb for the MM cohort. This was in contrast to a similar analysis in CLL (Lin*, et al* 2010) where purified tumour cells were analysed and individual telomere length distributions were significantly more homogeneous (mean SD = 1.28) compared to those observed in MM and MGUS patients ($p < .0001$; Mann-Whitney). The telomere length heterogeneity in the MM samples was consistent with the differing replicative histories of the numerous cell populations that composed the

unsorted bone marrow samples analysed.

The majority of telomere length profiles derived from MM patient samples displayed multi-modal distributions with sub-populations of cells displaying more extensive telomere erosion (highlighted in red in Figure 1A). To examine the telomere length distributions of purified myeloma plasma cell populations, we undertook cell sorting in a subset of myeloma bone marrow samples (n = 5) and analysed the $CD138^{+}$ and $CD138^{+}$ cell fractions separately. Distinct differences in the telomere length distributions were apparent (Figure 1B), with $CD138^{+}$ plasma cells (mean = 2.40kb) displaying significant telomere erosion compared to the $CD138^{-}$ cells (mean = 5.26kb; p = .008). These data indicate that the shorter telomere length distribution observed in unsorted MM samples represent $CD138^{+}$ malignant plasma cells. Importantly these data are consistent with MM plasma cells exhibiting an extensive replicative history that is distinct to that of bone marrow $CD138^{-}$ cells in these patients.

Overall, telomere length was shorter in MM compared to MGUS patient samples (P = .017; Mann-Whitney) with 19% of MM samples within the fusogenic range (Figure 1C); the telomere length threshold below which we detected telomere fusions in CLL (3.81kb)(Lin*, et al* 2014). Telomere length declined as a function of age in MM at rates consistent with the extensive literature on telomere dynamics in normal peripheral blood samples (-25 bp/year, p = .00056; Figure 1D)(Muezzinler*, et al* 2013). However, there was no significant difference in telomere length between any of the International Staging System (ISS) subgroups (p = .27; Figure 1C) or between sexes (p = .22).

The median telomere length of the MM cohort provided modest prognostic resolution (HR = 1.61 (1.04-2.53), p = .03; data not shown). In contrast, use of the previously determined telomere dysfunction threshold (Lin*, et al* 2014) was highly prognostic for overall survival in MM (HR = 3.42 (3.67-15.81), P < .0001; Figure 2A); a striking observation, given the variable contributions of plasma cells to the unsorted heterogeneous samples analysed.

Consistent with previous reports, the ISS provided strong prognostic information in our MM cohort (HR = 3.56 (2.92-9.13), p < .0001; Figure 2B), that was similar to that derived using the telomere dysfunction threshold. In order to assess whether telomere length could add prognostic resolution to the ISS, we performed multivariate analysis on 113/131 (86.3%) MM samples on which we had all relevant clinical data. In a model which included the potential covariates of mean telomere length, gender, age, ISS sub-groups and the telomere dysfunction threshold (3.81kb), the most important prognostic factor was ISS, followed by age and telomere length below 3.81kb (Table 1). After adjustment for ISS and age, telomere length <3.81kb was associated with significantly shorter survival (HR = 2.23 (1.26-3.96)**,** P = .006; Figure 2C). Despite the prognostic independence of telomere length, there was evidence of an interaction between ISS and this parameter, with the effect of short telomeres less prominent in patients with high risk ISS score (P = .05). However, in univariate analyses, stratified by ISS score, short telomeres still were associated with significantly worse survival in high risk ISS patients (P = .02). Overall, patients with good or standard risk ISS who manifested short telomeres, or high risk ISS patients with long telomeres

had intermediate survival when compared to concordant groups consisting of good/standard risk ISS and long telomeres and high risk ISS and short telomeres (Figure 2D). These findings suggest that a refinement of the risk classification could be obtained by incorporating telomere length assessment into the ISS for MM. Given the obvious differences observed between malignant plasma cells and other bone marrow constituents, we speculate that purification of the MM tumour cells would further enhance the clinical utility of high-resolution telomere length analysis in this disease.

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Conflict of Interest Statement:

SH declares no conflict of interests; REJ declares no conflict of interests; NHH 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; GHJ declares no conflict of interests; JMA declares no conflict of interests; GP declares no conflict of interests; CP 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.

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Table 1. Multivariate modelling revealed three significant independent variables.

Summary of Forward Selection					
Step	Effect entered	DF	Number In	Chi-Square test statistic	p-value
1	ISS 3	1	1	34.7751	<.0001
2	age	1	2	18.1900	<.0001
3	TL <3.81kb	1	3	7.7095	0.0055

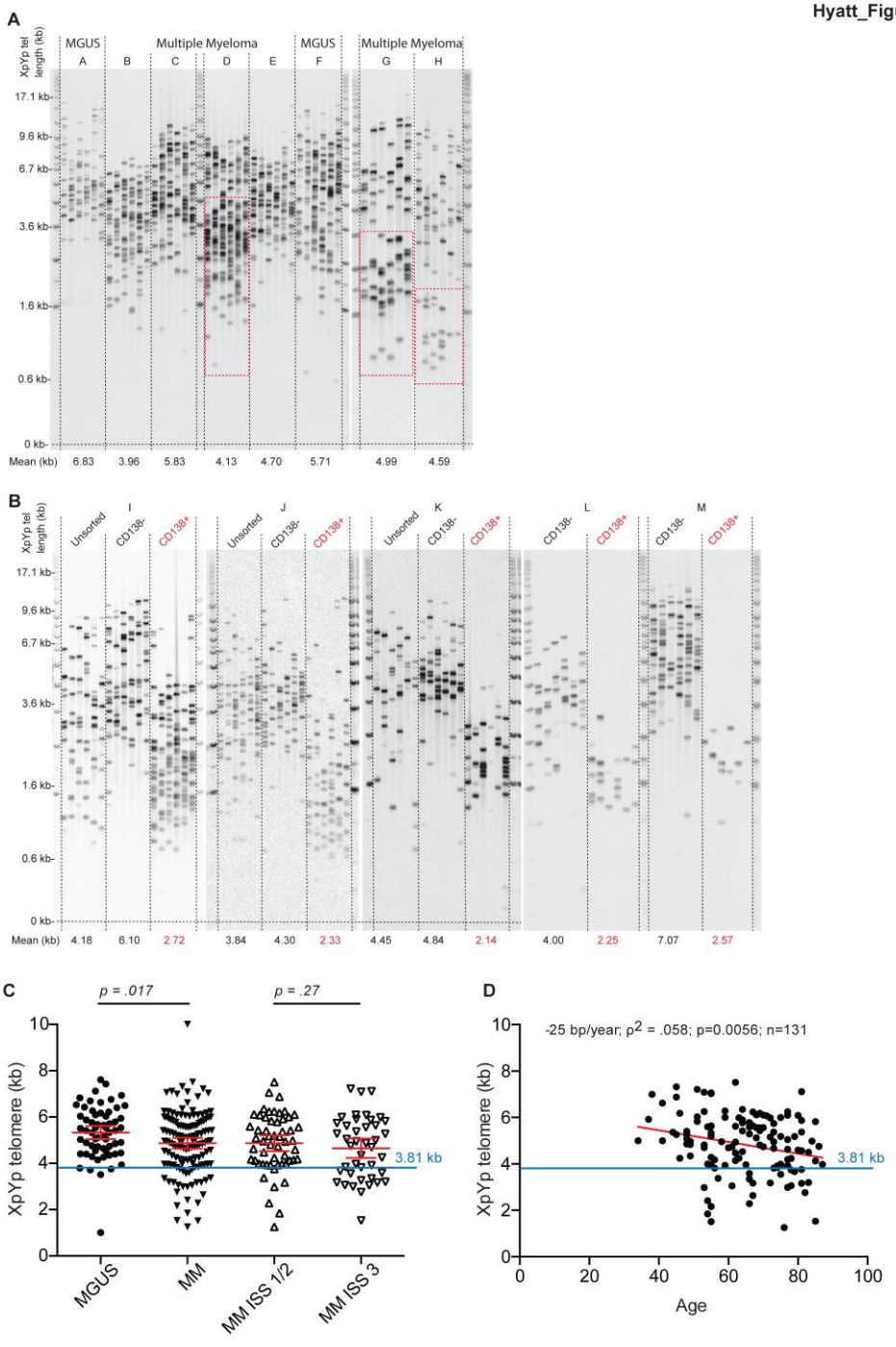
ISS 3: Multiple myeloma International Scoring System stage 3
TL <3.81kb: mean telomere length of less than 3.81kb
Using a Cox proportional hazards model with forward selection, only three variables were deemed to hold independent prognostic significance ($p \leq .05$).

Figure Legends

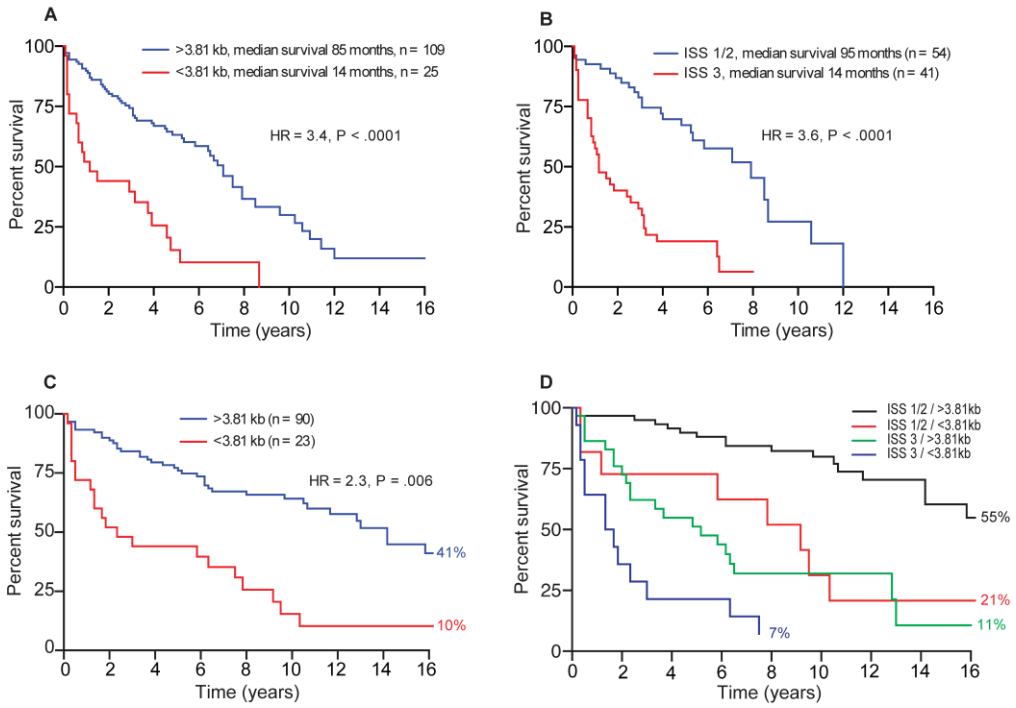
Figure 1. MM and MGUS exhibit heterogeneous telomere length profiles. (A) Examples of STELA of the XpYp telomere in 8 MM and MGUS samples. The mean of the telomere length profiles are indicated in black below each sample. Red boxes indicate the shorter modal telomere length profiles in samples that display a multimodal telomere length distributions. (B) Comparison of cell sorted $CD138^{+}$ and $CD138^{-}$ sub-populations revealed distinctly shorter telomere length profiles in $CD138^{+}$ cells. (C) Scatter plot depicting mean telomere length measurements for cohorts of MGUS and MM patients, and MM ISS 1/2 and ISS 3 sub-groups. The upper limit of telomere dysfunction (3.81kb) is shown as a blue horizontal line. Statistical comparisons where undertaken using non-parametric Mann-Whitney tests. (D) Plotting mean telomere length as a function of age shown in years. P value was determined using Spearman correlation.

Figure 2. Telomere length is highly prognostic in MM. Kaplan Meier survival analysis was performed on the MM cohort using the log-rank test (A) The telomere dysfunction threshold (<3.81kb) identified a subset of MM patients with inferior survival. (B) In keeping with previous reports, the ISS 3 sub-group also showed significantly inferior survival compared to the combined ISS 1/2 sub-group. (C) Even after adjustment for ISS and age, MM patients with telomere length <3.81kb had significantly shorter survival. (D) The combination of ISS and the telomere dysfunction threshold provided a refinement of their prognostic information. ISS 1/2 patients with short telomeres, or ISS 3 patients with long telomeres had intermediate survival when compared to concordant groups consisting of ISS 1/2 and long telomeres and ISS 3 and short telomeres.

Hyatt_Figure 1



Hyatt_Figure 2



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