Human Cancer Biology

Profiling Three-Dimensional Nuclear Telomeric Architecture of Myelodysplastic Syndromes and Acute Myeloid Leukemia Defines Patient Subgroups

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Abstract

Purpose: Myelodysplastic syndromes (MDS) are a group of disorders characterized by cytopenias, with a propensity for evolution into acute myeloid leukemias (AML). This transformation is driven by genomic instability, but mechanisms remain unknown. Telomere dysfunction might generate genomic instability leading to cytopenias and disease progression.

Experimental Design: We undertook a pilot study of 94 patients with MDS (56 patients) and AML (38 patients). The MDS cohort consisted of refractory cytopenia with multilineage dysplasia (32 cases), refractory anemia (12 cases), refractory anemia with excess of blasts (RAEB)1 (8 cases), RAEB2 (1 case), refractory anemia with ring sideroblasts (2 cases), and MDS with isolated del(5q) (1 case). The AML cohort was composed of AML-M4 (12 cases), AML-M2 (10 cases), AML-M5 (5 cases), AML-M0 (5 cases), AML-M1 (2 cases), AML-M4eo (1 case), and AML with multidysplasia-related changes (1 case). Three-dimensional quantitative FISH of telomeres was carried out on nuclei from bone marrow samples and analyzed using TeloView.

Results: We defined three-dimensional nuclear telomeric profiles on the basis of telomere numbers, telomeric aggregates, telomere signal intensities, nuclear volumes, and nuclear telomere distribution. Using these parameters, we blindly subdivided the MDS patients into nine subgroups and the AML patients into six subgroups. Each of the parameters showed significant differences between MDS and AML. Combining all parameters revealed significant differences between all subgroups. Three-dimensional telomeric profiles are linked to the evolution of telomere dysfunction, defining a model of progression from MDS to AML.

Conclusions: Our results show distinct three-dimensional telomeric profiles specific to patients with MDS and AML that help subgroup patients based on the severity of telomere dysfunction highlighted in the profiles. Clin Cancer Res; 18(12); 3293–304. ©2012 AACR.

Introduction

Myelodysplastic syndromes (MDS) are composed of a heterogeneous group of clonal disorders affecting hematopoietic progenitors that display genomic instability (1). MDS is characterized by dysplasia and ineffective hematopoiesis (2, 3). MDS represents I of the 5 major categories of myeloid neoplasms according to the classification of hematologic cancers by the World Health Organization (WHO; refs. 2–4). Myeloid neoplasms display a fundamental characteristic feature that is stem cell–derived clonal myelopoi-esis with altered proliferation and differentiation (3). MDS, de novo or secondary to ionizing radiation, toxins, or chemotherapeutic drug exposure, generally follows a course of progressive cytopenias with varying degrees of anemia, leukopenia, and thrombocytopenia. This renders the patients dependent on red cell transfusions or susceptible to infection or hemorrhage (2, 4). Pathologically, the 2
main characteristics of MDS are excessive intramedullary apoptosis of hematopoietic cells accompanied by excessive proliferation in the marrow (5, 6). This excessive apoptosis is largely cytokine-mediated, with a number of proinflammatory cytokines being overexpressed in the marrows, such as TNF, TGFβ, and IL1β. This phenomenon is related to the nonspecific haploinsufficiency of RP14 (7–10). However, the cellular and molecular mechanisms of the cytopenias are not well understood.

Importantly, 1 of 3 of cases of MDS is characterized by progression to acute myeloid leukemia (AML/MDS), by the accumulation of genetic abnormalities, and blockage of cell differentiation and accumulation of blast cells. The pathology of MDS and AML/MDS are cytologically distinguishable because the cell types show very different morphologies, including their dysplastic features. The evolution of MDS to AML/MDS is characterized by genomic instability (11, 12). AML is a clonal expansion of myeloid blasts in bone marrow, blood, or other tissues, and is distinguished from MDS by the increased numbers of blast cells (more than 20% of blast cells in the marrow). MDS and AML are sustained by genomic instability, which is a hallmark of all cancers (11, 13). Although chromosomal aberrations are common in both MDS and AML, a substantial proportion of cases have normal karyotypes (12).

One major cause of genomic instability and cellular apoptosis is telomere dysfunction. However, few studies have addressed the impact of telomeres in MDS, AML/MDS and AML (11, 14). Telomeres are the ends of chromosomes capped by a protective protein complex, termed shelterin (15). Proper telomere capping preserves chromosomal integrity and prevents terminal end-to-end fusions. Telomere loss or dysfunction results in breakage-bridge-fusion cycles, aneuploidy, and ongoing chromosomal rearrangements (12). Telomeres also are thought to contribute to chromosome positioning within the nucleus (16). The three-dimensional (3D) nuclear organization of telomeres allows for a distinction between normal and tumor cells: nuclei of the latter tend to be disorganized and commonly contain telomeric aggregates (17). Alterations in telomere architecture and telomeric dysfunction are associated with the onset of genomic instability (18, 19, 20). Our previous work showed altered 3D telomeric organization in different types of cancer (12, 17, 19, 20–25). However, despite several studies showing the role of telomere dysfunction in the occurrence of hematopoietic malignancies, little is known about their possible role(s) in the evolution of MDS and transition to AML/MDS (26). Data describing the average telomere length and individual telomere length in MDS and AML showed that the transition from MDS to AML was characterized by increased telomere shortening and chromosomal abnormalities (11, 14, 27–30). However, the underlying mechanism(s) remain unknown. To decipher cellular and molecular mechanisms in MDS and AML, we examined telomere dysfunction and alterations in the 3D nuclear telomere architecture.

Materials and Methods

Patients

This study received approval by the Research Ethics Board on human studies at the University of Manitoba (H2010:277) and in Brazil (1735/2011). Patients were enrolled at regular basis during their clinical follow-up after informed consent and before any treatment. The patient population included in this study was composed of 7 patients from Cancer Care Manitoba (CCMB) and 87 patients from Brazil. In this latter country, all patients’ samples were sent to the Hematology Service of the University Hospital, School of Medicine of Ribeirão Preto, University of São Paulo. The diagnosis of MDS and AML was based on morphology, immunophenotypic analysis and cytogentic. The patients enrolled for this investigation had no previous diagnosis of other hematologic diseases. The diagnosis and clinical management of our cohort patients were made according to their clinical presentation on the basis of clinical features, pathologic, immunophenotypic, cytologic, and cyto genetic diagnoses as recommend by the WHO.

Clinical surrogates, French-American-British and WHO classifications

Clinical data were collected from a total of 94 patients from Brazil and Manitoba following their enrolment in this study between 2006–2011 (Brazil) and 2010–2011 (Mani toba). Only patients with suspicion of MDS or AML were included in this cohort before any treatment. According to WHO criteria based on clinical, pathologic and cytogenetic diagnoses, our cohort was composed of 56 cases of MDS and 38 cases of AML. The MDS cohort consisted of refractory cytopenia with multilineage dysplasia (RCMD = 32 cases), refractory anemia (RA = 12 cases), refractory anemia with excess of blasts 1 and 2 (RAEB-1 = 8; RAEB-2 = 1 case), refractory anemia with ring sideroblasts (RARS = 2 cases), and MDS associated with isolated del(5q) (1 case). The AML cohort was composed of AML-M4 (12 cases), AML-M2 (10 cases), AML-M5 (5 cases), AML-M0 (5 cases), AML-M1 (12 cases), MDS associated with isolated del(5q) (1 case). The AML cohort was composed of AML-M4 (12 cases), AML-M2 (10 cases), AML-M5 (5 cases), AML-M0 (5 cases), AML-M1 (12 cases), MDS associated with isolated del(5q) (1 case).
(2 cases), AML-M4eo (1 case), and AML with multidysplasia-related changes (1 case; Supplementary Tables S1 and S2). The mean age of the patients was 58 years (range 17–88 years) and the median age 62 years (range 17–88 years; Supplementary Tables S1 and S2).

Sampling
Bone marrow aspiration was carried out from each patient following standard clinical procedure and sent to the hematologic laboratory, to the cytogenetic laboratory for clinical and pathologic diagnosis, and to our research laboratory for the analysis of the 3D nuclear telomeric architecture. Cytologic diagnosis and the cytogenetic determination of chromosomal abnormalities were assessed according to standard protocols (Supplementary Tables S1 and S2). Samples from Brazil were 3D fixed before shipment to the Manitoba Institute of Cell Biology (MICB), where the samples received from CCMB were 3D fixed. Three-dimensional fixation was carried out according to our standard methods (20, 25).

Three-dimensional quantitative FISH
The quantitative FISH (QFISH) procedure was carried out as described previously (20, 25). Briefly, after incubation in 3.7% formaldehyde/1× PBS for 20 minutes, slides were immersed in 20% glycerol/1× PBS for 1 hour. Then, the nuclei were treated by 4 repeated cycles of freeze-thaw in glycerol/liquid nitrogen. The next step was equilibration of the slides in 0.1 HCl solution and fixation in 70% formamide/2× SSC for 1 hour. Immediately after this, 8 μL of PNA telomeric probe (Dako) was applied to the slides, which were sealed and placed into a Hybrite (Vysis; Abbott Diagnostics). Denaturation was carried out at 80°C for 3 minutes for both cells and probe, followed by their hybridization at 30°C for 2 hours. The slides were then washed twice for 15 minutes in 70% formamide/10 mmol/L Tris pH 7.4 followed by washing in 1× PBS at room temperature for 1 minute while shaking and in 0.1× SSC at 55°C for 5 minutes while shaking. Furthermore, the slides were washed in 2× SSC/0.05% Tween 20 twice for 5 minutes each at room temperature while shaking. Thereafter, the slides were counterstained with 4',6-diamino-2-phenylindole (DAPI; 0.1 μg/mL) and cover slipped with ProLong GOLD antifade reagent (Invitrogen, Molecular probes) before image acquisition.

Image acquisition and three-dimensional image analysis using TeloView system
We carried out 3D image analysis on 30 interphase nuclei per slide using an AxioImager Z1 microscope (Carl Zeiss Canada Ltd) and an AxioCam HRm charge-coupled device (Carl Zeiss Canada Ltd.). A 63× oil objective lens (Carl Zeiss Canada Ltd.) was used at acquisition times of 600 milliseconds for Cy3 (telomeres) and 7 milliseconds for DAPI (nuclei). Sixty z-stacks were acquired at a sampling distance of x/y: 102 nm and z: 200 nm for each slice of the stack. AxioVision 4.8 software (Carl Zeiss Canada Ltd.) was used for 3D image acquisition, and a constrained iterative algorithm (31, 32) was used for deconvolution. Deconvolved images were converted into TIFF files and exported for 3D analysis using the TeloView software program (refs. 31, 32; Fig. 1A and B).

Data analysis
TeloView (1, 20, 25) computes 5 parameters on each sample:

1. the number of signals, that is, the number of telomeres.
2. the signal intensity, that is, the telomere length.
3. the number of telomere aggregates, which means clusters of telomeres that are found in close proximity that cannot be further resolved as separate entities by TeloView at an optical resolution limit of 200 nm.
4. the a/c ratios determined by representing the nuclear space occupied by the telomeres as an ovoid, with 2 main axes, a and b, that are equal in length, and a third axis, c, that has a different length (31). This distribution of telomeres in the 3D space of the nucleus varies with cell cycle; as the specific stages of the cell cycle (G0/G1, S, and G2) phases have characteristic a/c ratios, one can determine where they reside in the cell cycle (31). The a/c ratio is a means of defining progression through cell cycle in interphase cells (25).
5. the distance of each telomere from the nuclear center versus the periphery.

These parameters in a 3D nucleus define all 3D telomere features we measured.

Three types of histograms were produced: (i) line graphs showing the distribution in the intensity of the acquired telomere fluorescent signals; (ii) histograms of the distribution of the number of aggregates per cell; and (iii) histograms of the distribution of the number of acquired signals per cell. The percentage of cells having telomeric aggregates, the mean number of signals, and mean number of aggregates per cell was calculated. The histogram data from different samples were combined into a single chart for comparison.

Statistical considerations
Fifteen subgroups were defined on the basis of their 3D telomeric profiles. The telomeric parameters (number, length, telomere aggregates, nuclear volumes, and a/c ratio) were compared between these 15 subgroups using a nested factorial ANOVA. Multiple pairwise comparisons using a least square mean tests followed a significant omnibus subgroup effect.

Distribution of telomere intensities in MDS and AML clinical categories was compared between various patient subgroups using χ2 contingency analysis. Cell parameter averages were analyzed between subgroups with nested factorial analysis of variance taking both patient and cellular variations into account. Significance level was set at α = 0.05.
Classification of the different clinical subgroups of patients according to the number of telomeres (signals), telomere lengths (intensities), and the number of telomeric aggregates were determined by stepwise discriminate analyses.

Results

Three-dimensional nuclear profiling using the number of telomeres, nuclear distribution of telomeres, signal intensities, and telomeres aggregates

All samples were analyzed in a blinded fashion. We first analyzed the 3D telomere profiles (Fig. 1C), that is, the total number of telomeres versus their lengths and aggregates in each cell of each sample using TeloView (20, 25, 31). Subsequently, the 3D telomere profile was defined by the distribution pattern of telomeres per sample according to their intensity (length of telomeres) for one sample; this 3D telomere distribution is one part of the 3D telomeric profile. Red bars separate the three-cell populations with short, intermediate, and long telomeres, respectively. (a.u.), arbitrary units.

Figure 1. A, 2D and B, 3D view of QFISH of bone marrow cell nucleus (blue) and telomeres (red); C, graph distribution of number of telomeres according to their intensity (length of telomeres) for one sample; this 3D telomere distribution is one part of the 3D telomeric profile. Red bars separate the three-cell populations with short, intermediate, and long telomeres, respectively. (a.u.), arbitrary units.
differences between the MDS subgroups and the AML sub-
groups ($P < 0.001$). Each of the quantitative telomere 
parameters exhibited significant differences between MDS and AML. Furthermore, statistical analyses combining all 3D telomere parameters revealed significant differences between all subgroups ($P < 0.05$; Supplementary Table S5).

Studying the dynamic variation of the number of telomeres versus the telomere intensities and the number of telomere aggregates, we suggest a chronologic evolution between these different subgroups (Fig. 2 and Fig. 3, Supplementary Fig. S1 and Table S5). In fact, these profiles seemed linked to the evolution of increased telomere dysfunction in these subgroups. We suggest that the evolution of MDS progresses from low to high level of telomere dysfunction, that is, from early stage of MDS to more severe stages followed by the transformation to AML (Fig. 2 and Fig. 3; Fig. 4). Future studies into this process are warranted.

Three-dimensional telomere profiles versus chromosomal abnormalities in the fifteen subgroups

To evaluate the chromosomal abnormalities in the 15 blindly examined subgroups, the code was removed and patients were placed into their respective disease groups. We compared the cytogenetic data in the 15 subgroups (Table 1), and we found patients with distinct cytogenetic aberrations, and patients sharing identical chromosomal abnormalities in all groups (Table 1; Fig. 4), as well as patients with normal karyotypes. Overall, the cytogenetic features of each patient and group did not indicate cumulative acquisitions of chromosomal abnormalities from one subgroup to the following one.
We concluded that the cytogenetic analyses of the samples did not discriminate the patient subgroups (Fig. 4). This is in contrast to their definition based on the 3D nuclear telomeric profiles (Table 1; Fig. 4).

### Three-dimensional telomere parameters in MDS and AML

We analyzed the telomere parameters between patients with clinical diagnoses of MDS and AML (Table 2). Number of signals per nucleus, telomere aggregates per nucleus, total telomere intensity, and average intensity of signals were all higher in the AML cases than in the MDS cases ($P < 0.0001$ for all comparisons, except the average intensity of signal for which $P > 0.05$).

We concluded that telomere parameters are highly accurate to distinguish MDS to AML. This reinforced our model of applying the 3D telomeric profiles for the classification of both MDS and AML (Figs. 2 and 3; Supplementary Fig. S1 and Table S5).

### Three-dimensional telomere parameters versus cytogenetic risk factor classifications of AML

The WHO classification of AML takes into account the cytogenetic and molecular data to predict the clinical outcome of AML patients following treatment. Eight cases of our AML patients fall into the “favorable” category, 12 into “intermediate,” and 5 into “unfavorable.” 9 into “AML not otherwise specified,” and 4 lacked karyotype information. We compared the 3D telomeric profiles of each WHO group of patients to the other groups and vice versa. Comparing the telomere parameters between the “favorable,” “Intermediate,” and “unfavorable” categories, only the number of signals per nucleus displayed a difference, with the “unfavorable” cases having an average 35% more signals than the “favorable” cases ($P = 0.03$). All other comparative pairing analyses using these parameters were not significant (Supplementary Table S3). However, according to 3D telomere profiles, the 6 AML subgroups were different from WHO classes. We concluded that future validation with larger patient cohorts is needed to establish our 3D telomeric profiling as an additional tool for clinical management in AML. Notwithstanding, these results reflect a relative cytologic homogeneity of the categories that might be linked to the increased number of blast cells. Indeed, AML is characterized by increasing number of blasts because of the hiatus of maturation that avoids the differentiation of precursor cells. The number of blasts in AML can vary from 20 to 70 and to finally reach 100%. At this latter level, maybe all the blasts cell display the same 3D telomere profile, paving the way of a possible link between number of blast and telomere dysfunction. Indeed, telomere dysfunction is a continuum with increased genomic changes as is the continuing increased number of blasts. We concluded that the cytogenetic categorizations from WHO classification of AML did not reflect the genomic instability in AML as observed using the 3D telomeric profiles (Figs. 2 and 3; Table 2; and Supplementary Fig. S1 and Table S5).

### Three-dimensional telomere parameters compared with IPSS and WPSS in MDS patients

Using the defined indexes such as IPSS (International Prognostic Scoring System) and WPSS (WHO Prognostic Scoring System; refs. 33, 34) to establish the prognostic indexes of the follow-up of MDS patients, we classified our patients and compared these indexes with aforementioned telomere parameters. Using IPSS, our MDS patients fall into...
3 groups: IPSS less than 2 (47 cases); IPSS more than 2 with age less than 60 years (3 cases); and IPSS more than 2 with age more than 60 years (6 cases). Comparative statistical analyses using the different telomere parameters between these 3 groups showed no significant differences ($P > 0.05$; Supplementary Table S4A).

Furthermore, all our MDS patients fall in 4 groups using WPSS: WPSS $= 0$ (43 cases); WPSS $= 1$ (12 cases); WPSS $= 2$ (1 case); and WPSS $= 3$ (1 case). Comparative statistical analyses using the different aforementioned telomere parameters between these 4 groups showed no significant differences, except the average intensity of all the telomere signals between both groups in the WPSS $= 0$ and WPSS $= 1$ ($P = 0.03$; Supplementary Table S4B). We found that the prognostic indexes such as IPSS and WPSS did not discriminate MDS patients although this was achieved using their 3D nuclear telomeric profiles (Supplementary Table S4; Figs. 2 and 3; Table 1).

**Three-dimensional telomeres and cell-cycle distribution in MDS and AML.**

We next investigated nuclear volumes, the distribution of the telomeres per nuclear volume, and the $a/c$ ratio. These 3 parameters allow for the characterization of cell-cycle distribution, similar to Ki67 (25), cell size, and overall distribution of telomeres within the 3D nuclear space.

The nuclear volume is significantly higher in AML than MDS ($P < 0.0001$), which likely might be attributable to the increased number of blast cells in AML. This early process of transformation of MDS to AML is detected by the variation in the nuclear volume. Furthermore, this latter is significantly different between WHO AML classes (all $P < 0.05$). This reinforces the value of the increased numbers of blast cells in the prognostic course of AML that our tool can detect very early.

We evaluated whether there was a difference in cell cycle between our patients with MDS and AML. Although the $a/c$ ratio in AML patients was somewhat higher, this did not achieve significance (Table 2). In line with the $a/c$ ratio, telomere distribution per nuclear volume did not show any significant difference between MDS and AML. This might be related to a similar cycling state of cells in both diseases, in which cells are in a proliferative state.

Finally, in MDS subgroups defined by the IPSS or the WPSS, no significant difference was found in any of the 3 aforementioned parameters (Supplementary Table S4). This is in line with the fact that the IPSS and the WPSS do not reflect the cell cycle of MDS, nor its proliferative index.

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Table 1. Patients subgroups according to 3D telomeric profiles and their cytogenetic data

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Discussion

We analyzed the 3D nuclear telomere architecture and determined the telomere numbers, the presence of telomere aggregates, telomere signal intensities, nuclear volumes, and nuclear telomere distribution in MDS and AML. Using these parameters, we determined 3D telomeric profiles, and we were able to blindly subdivide the MDS patients into 9 subgroups and the AML patients into 6 subgroups. WHO classifications also distinguish 8 classes of MDS and 4 major classes (regrouping many entities in one class) of AML (2–4).

Analyzing the evolution of telomere dysfunction in MDS and AML allows us to propose a model of telomere dysfunction in the progression from MDS to AML. Our results show distinct telomeric profiles specific to patients with MDS and AML and imply, for the first time, a chronicologic and evolutionary process of telomere dysfunction in both diseases. Our data are consistent with the hypothesis that the telomere dysfunction generates genomic instability, leading to worsening cytopenias and to the transformation of MDS to AML. Three-dimensional telomere profiles seem to be a potential biomarker that, in the future, may improve the clinical management of these patients.

Table 1. Patients subgroups according to 3D telomeric profiles and their cytogenetic data (Cont’d)

<table>
<thead>
<tr>
<th>Telomere profile subgroups</th>
<th>Patients</th>
<th>Cytogenetic results</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>P64</td>
<td>45,XY,inv(16)(p13q22)[12]/46,XY[8]</td>
</tr>
<tr>
<td></td>
<td>P57</td>
<td>46,XX,del(6)(q15q23)[6],inv(16)(p13q22)[4]/46,XX[10]</td>
</tr>
<tr>
<td></td>
<td>P82</td>
<td>46,XX,t(8;21)(q22;q22)[8]/46,XX[11]</td>
</tr>
<tr>
<td></td>
<td>P90</td>
<td>47,XX,t(8;21)(q22;q22),+l(17)(q10)[4]/46,XX[16]</td>
</tr>
<tr>
<td></td>
<td>P81</td>
<td>nuc ish(AML1 × 2),[ETO × 2],(AML1 con ETO x 1)[167/200]</td>
</tr>
<tr>
<td></td>
<td>P60</td>
<td>46,XX[16]</td>
</tr>
<tr>
<td></td>
<td>P83</td>
<td>46,XX[20]</td>
</tr>
<tr>
<td></td>
<td>P78</td>
<td>45,X,-Y[3],t(8;21)(q22;q22)[12]/46,XY[8]</td>
</tr>
<tr>
<td></td>
<td>P90</td>
<td>No metaphase available</td>
</tr>
<tr>
<td></td>
<td>P86</td>
<td>46,XY[14]</td>
</tr>
<tr>
<td></td>
<td>P62</td>
<td>No metaphase available</td>
</tr>
<tr>
<td></td>
<td>P70</td>
<td>46,XX[18]</td>
</tr>
<tr>
<td></td>
<td>P73</td>
<td>45,XX,inv(16)(p13q22)[8]/46,XY[9]</td>
</tr>
<tr>
<td></td>
<td>P88</td>
<td>47,XY,+4[10]/46,XY[10]</td>
</tr>
<tr>
<td></td>
<td>P65</td>
<td>46,XY[20]</td>
</tr>
<tr>
<td></td>
<td>P67</td>
<td>45,XY,-4[12]/46,XY[8]</td>
</tr>
</tbody>
</table>

Three-dimensional telomere profiles are indicative of apoptosis and proliferation in MDS

In all subtypes of MDS, high rates of apoptosis mediated by inflammatory cytokines in the bone marrow contribute to the ineffective hematopoiesis and peripheral cytopenias (8, 10, 35). However, apoptosis is not inherited uniformly by all the cell lineages in the marrow, as the cells most sensitive to apoptosis are the ones undergoing premature death in the marrow, whereas those most resistant to it are the cells that make it into the blood (35, 36). This assertion is strongly supported by the fact that granulocytes from normal healthy donors are more sensitive to apoptosis than the clonal granulocytes in MDS (36). The initial clonal expansion and the selective apoptosis of 3 different cell lineages in the marrow remain to be understood. Haploinsufficiency of both RPS14 and RBM22 genes may contribute to this phenomenon in the pathogenesis of the 5q-syndrome (3), but the mechanisms in other subtypes of MDS remain to be elucidated.

One major causal phenomenon producing apoptosis and genomic instability is telomere dysfunction (12). Telomere dysfunction is tissue and cell type specific and may explain the occurrence of both proliferation and apoptosis in specific cell lineages. In MDS, cytopenias affect 3 cell lineages (erythroid, granulocytic, and megakaryocytic lineages). Taking into account that the granulocytic cell lineage is more resistant to apoptosis, the fact that anemia is most often the first manifestation of marrow failure in MDS might be explained by apoptosis due to telomere dysfunction in this specific cell lineage. This also might explain the abnormalities in the megakaryocytic lineage. In the end, telomere dysfunction might prevent maturation in the granulocytic lineage, which resists apoptosis and maintains this cell lineage at the level of blast cells. The result of this phenomenon is the increase of blast cells and the transformation of MDS to AML. Telomere dysfunction might generate proliferation in one cell lineage (cells in which genomic instability gives a proliferative advantage) and apoptosis in another cell lineage (cells in which genomic instability leads to cell death) at the same time. It may thus explain the clonal expansion and the selective apoptosis in the bone marrow. A suitable assessment of telomere dynamics in MDS and AML might explain the pathogenesis of both diseases (27, 28). Taking these findings into account, a chronologic and evolutionary process of telomere dysfunction in both diseases might be linked to worsening cytopenias and to the transformation of MDS to AML.

Three-dimensional telomere profiles define MDS and AML subgroups

Using TeloView to evaluate telomere dynamics in every cell, we show for the first time different 3D telomeric profiles specific to subgroups of MDS and AML (Figs. 2 and 3; Supplementary Fig. S1). These profiles follow an apparently sequential progression, consistent with a working model of telomere changes as drivers of evolution of MDS to AML (Fig. 4). It will therefore be important to
investigate with larger patient cohorts whether these telomere profiles can serve as biomarker of MDS and AML.

Although we have not examined a longitudinal profile of single patients, our data set describes the existence of MDS- and AML-specific 3D nuclear telomere dysfunction. Using our 3D nuclear parameters blindly, we were able to subgroup the patients into MDS and AML and to subdivide these disease groups further. Future analyses will have to provide evidence on a patient-by-patient basis as to whether 3D nuclear telomere dysfunction can serve as reliable biomarker in predicting disease progression. Indeed, our previous work has shown that one key difference between normal and cancer cells is the presence of telomere aggregates. We found telomere aggregates in all the samples and telomere dysfunction in both diseases, but with a significant difference between MDS and AML (Table 2; P < 0.0001). This might allow for the detection of the early stages of MDS in patients presenting with anemia because changes in nuclear architecture precede the development of chromosomal abnormalities. It was shown that telomere shortening in MDS is independent of the presence of cytogenetic abnormality.

Nuclear telomere profiling seems to be more accurate to reflect genomic instability underlying MDS and AML than French-American-British classification of both diseases. Further analyses according to the WHO classification are in line with that, despite the consideration of cytogenetic and molecular abnormalities in MDS and AML patients. However, all these clinical classes of AML show genomic instability that we profiled accurately. Three-dimensional nuclear telomeric profiles are the direct reflection of chromosomal abnormalities, rather than clinical response to treatment grouping. Telomere-associated genomic instability might, in the future, be a better indicator of the clinical outcome of MDS/AML. This hypothesis, of course, will need to be validated in a prospective clinical trial.

Comparison of 3D telomeres profiles with the IPSS and the WPSS in MDS

Analyses of MDS patients in parallel to the available prognostic markers IPSS and WPSS led to the conclusion that both indexes did not discriminate MDS patients although this was achieved using their 3D nuclear telomeric profiles. Indeed, IPSS, which is based on percent marrow blasts, type of cytogenetic abnormalities, and degree and number of cytopenias, and WPSS, which adds to IPSS number of blood transfusions, both predict survival and evolution to acute leukemia characterized by increased genomic instability. We propose that monitoring 3D telomere dysfunction might be a very powerful marker to measure this transformation. Furthermore, it may be a better indicator of therapeutic response because an optimal response will lead to a normal cellular biology, including elimination of abnormal telomeric aggregates and the increase of normal 3D telomeric profiles.

Table 2. Three-dimensional telomere parameters according to clinical diagnosis

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Total number of signals Mean (SD)</th>
<th>Total number of aggregates Mean (SD)</th>
<th>Total intensity Mean (SD)</th>
<th>Average intensity of all signals Mean (SD)</th>
<th>a/c ratio Mean (SD)</th>
<th>Nuclear volume Mean (SD)</th>
<th>Telomere per nuclear volume Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDS</td>
<td>20.10 (8.17)</td>
<td>1.81 (1.56)</td>
<td>307,567 (137,676)</td>
<td>15,740 (5,237)</td>
<td>9.65 (6.21)</td>
<td>221,969 (139,522)</td>
<td>0.11 (0.05)</td>
</tr>
<tr>
<td>AML</td>
<td>31.10 (13.09)</td>
<td>3.32 (2.46)</td>
<td>523,641 (245,868)</td>
<td>17,504 (6,513)</td>
<td>14.35 (7.86)</td>
<td>316,428 (187,906)</td>
<td>0.11 (0.05)</td>
</tr>
<tr>
<td>P value between MDS and AML</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.02</td>
<td>&lt;0.0001</td>
<td>0.48</td>
<td></td>
</tr>
</tbody>
</table>

Three-dimensional telomere profiles as a potential biomarker of genomic instability in MDS and AML

MDS and AML both undergo a dynamic process of genomic instability, which may be monitored by the 3D analyses of telomeres. Combinatorial analyses of these 3D nuclear telomere parameters versus different classification of MDS and AML strengthen the validity of our telomeric profiles for the classification of MDS versus AML and describe patient subgroups in both. Notwithstanding, due to the difficulties to obtain 3D-preserved patient samples at different time points of the disease and to have a longitudinal follow-up of the patients due to slow evolution of MDS in several cases, we are beginning to investigate an animal model of MDS/AML to follow disease progression and 3D telomere dysfunction during this process. C57BL/6-Tg(Vav1-NUP98/HOXD13) G2Apla/J hemizygote mice develop MDS with peripheral blood cytopenia and dysplasia and normocellular to hypercellular bone marrow. By 14 months of age a subset of hemizygotes succumbs to malignant acute myeloid leukemia or severe anemia and leukopenia. This mutant mouse strain may be useful in studies of MDS and leukemia and to validate our 3D nuclear telomeric profiling of both diseases.

Conclusion

Our results show significant differences in the 3D nuclear telomeric organization between MDS and AML in terms of
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Gadji, S. Mai

Development of methodology: M. Gadji, S. Mai, F.M. de Oliveira

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc): M. Gadji, I.A. Awe, P. Rodrigues, R. Kumar, D.S. Houston, L. Klewes, E.M. Rego, R.F. Passetto, F.M. de Oliveira

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Gadji, I.A. Awe, P. Rodrigues, E.M. Rego, S. Mai

Writing, review, and/or revision of the manuscript: M. Gadji, I.A. Awe, R. Kumar, T.N. Dieye, R.F. Passetto, F.M. de Oliveira, S. Mai

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