Telomere Dynamics in Myelodysplastic Syndrome Determined by Telomere Measurement of Marrow Metaphases

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ABSTRACT

Myelodysplastic syndrome (MDS), which is known to be a preleukemic state, is a heterogeneous entity characterized by ineffective hematopoiesis and dysplastic morphological features. Most MDS patients show erosive telomeric repeats (TTAGGG)n, without up-regulation of telomerase activity, suggesting that telomere shortening may be linked to cellular senescence in MDS. We measured telomere length in samples from 13 MDS patients and 8 healthy volunteers, based on telomere signals of individual chromosomes, using digital images of metaphases after quantitative fluorescence in situ hybridization (Q-FISH) with peptide nucleic acid probes and compared the results with results obtained with the standard method of determining terminal restriction fragment (TRF) length. In healthy volunteers, we found a significant correlation between TRF length and telomere fluorescence signals detected by Q-FISH, and a relatively wide distribution of fluorescence telomere signals was demonstrated in every sample. In contrast, we found no linear correlation between TRF length and telomere fluorescence signals in MDS, and most MDS patients showed weak telomere fluorescence signals, corresponding to short telomeres, with a narrow range compared with normal subjects. TRF length represented telomere DNA in whole marrow cells, whereas telomere fluorescence signals by Q-FISH represented only marrow metaphases corresponding to MDS-derived cells. Metaphases from most MDS patients showed homogeneous telomere shortening, irrespective of the presence of cytogenetic abnormality. In contrast, marrow metaphases from normal individuals showed a relatively wide range of telomere signals in each metaphase, indicating that in MDS cells, telomere shortening mechanisms that normally exist might be dysregulated. Therefore, analysis of telomere distribution as well as average telomere length detected by Q-FISH might be useful to clarify the telomere dynamics of MDS cells.

INTRODUCTION

Telomeres are the repetitive sequences (TTAGGG)n on the ends of chromosomes and are maintained in equilibrium at an organ-specific length (1–3). Loss of telomere length leads to chromosome end-to-end fusion, chromosome rearrangements, and instability (4, 5). Telomere shortening results from cell division and leads to genomic instability and cancer progression (3). Up-regulation of telomerase, a ribonucleoprotein enzyme responsible for complete cell replication with shortened telomeres, is detectable in at least 85% of cancers (6, 7).

Average telomere length is conventionally assessed by Southern blot analysis and is represented as TRF3 length (1, 2). This method requires a large number of cells (>10⁵) without information on the individual telomere lengths in the cells. Moreover, TRF length represents the telomere lengths of all cells in the sample, including contaminating cells, irrespective of whether they enter the cell cycle. PNAs are pseudo-peptides that can hybridize to a counterpart DNA or RNA with higher affinity and specificity (8). Recently, measurement of the telomere lengths of individual chromosomes from digital images of metaphases after Q-FISH with PNA probes has been proposed (9–11). The merit of Q-FISH is that it reveals the exact telomere signals that may correspond to the telomere length of each chromosome and can detect differences in telomere signals among cells. In contrast to Southern blot analysis, however, Q-FISH detects telomere signals in a limited number of metaphase cells.

Most MDS patients do not show marked up-regulation of telomerase activity, suggesting that telomere shortening rather than telomerase up-regulation may reflect the telomere dynamics in MDS (12–15). It has been demonstrated that TRF in MDS patients does not show progressive telomere shortening dependent on aging, unlike marrow cells in normal healthy volunteers. To obtain further insight into the telomere dynamics of MDS precisely, we used PNA probes to detect telomere length at the ends of chromosomes.
single cell level and compared the results of Southern blot and Q-FISH analysis. Because bone marrow cells obtained from MDS patients consist of different mature hematopoietic cells and may include normal background cells, we thought that Q-FISH is suitable to determine exact telomere erosion in each MDS cell.

**MATERIALS AND METHODS**

**Patients and Healthy Volunteers.** The diagnosis of MDS was based on the French-American-British classification (16). The distribution of different French-American-British subtypes was refractory anemia (RA; \( n = 7 \)), RA with excess blasts (RAEB; \( n = 3 \)), and RAEB in transformation (RAEBt; \( n = 3 \)). The patients’ clinical characteristics are shown in Table 1. The median age of the patients was 55 years (range, 29–86 years). Bone marrow aspirates were obtained at the time of MDS diagnosis and were used to determine telomere length as described below. Bone marrow aspirates were also obtained from 8 healthy volunteers. The median age of the healthy volunteers was 55 years (range, 29–68 years). There was no significant difference in average age between the MDS patients and the healthy volunteers (56.9 vs. 49.7 years; \( P = 0.329 \)). All samples from patients and healthy volunteers were acquired after obtaining their informed consent. We also used the established leukemia cell lines K562 and OM9;22 (17, 18) for this study.

Cytogenetic analyses were carried out by 24-h culture of bone marrow cells without any mitogens, and the conventional Q-banded technique was performed. Chromosome abnormalities were described according to the International System for Human Cytogenetic Nomenclature (19). We used marrow cells fixed with Carnoy’s solution for Q-FISH analysis as described below.

**Table 1** Clinical characteristics and results of TRF and Q-FISH analysis in MDS patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Survival (months)</th>
<th>TRF (kb)</th>
<th>Expected TRF (kb) by Q-FISH</th>
<th>Abnormal/undetectable/countable metaphase*</th>
<th>Karyotype (bone marrow cells) by Q-banded analysis</th>
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</thead>
<tbody>
<tr>
<td>101</td>
<td>M</td>
<td>29</td>
<td>RA</td>
<td>5</td>
<td>3.2</td>
<td>3.6</td>
<td>5/0/5</td>
<td>46,XY [1]/46,XY,del(5)(q13q34),+8,t(19;21)(p12q11),–20 [19]</td>
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<tr>
<td>102</td>
<td>M</td>
<td>43</td>
<td>RA</td>
<td>89+</td>
<td>6.5</td>
<td>4.2</td>
<td>2/3/5</td>
<td>48,XY,+8,+16 [12]/48,XY,+del(1)(p21),+8 [5]</td>
</tr>
<tr>
<td>103</td>
<td>M</td>
<td>55</td>
<td>RA</td>
<td>32+</td>
<td>8.8</td>
<td>4.1</td>
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<tr>
<td>104</td>
<td>M</td>
<td>33</td>
<td>RA</td>
<td>28</td>
<td>9.0</td>
<td>3.6</td>
<td>3/2/5</td>
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<tr>
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<td>RA</td>
<td>25+</td>
<td>9.2</td>
<td>4.4</td>
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<td>M</td>
<td>67</td>
<td>RA</td>
<td>9</td>
<td>9.3</td>
<td>4.6</td>
<td>3/3/6</td>
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<tr>
<td>107</td>
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<td>RA</td>
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<td>46,XY [20]</td>
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<tr>
<td>108</td>
<td>F</td>
<td>33</td>
<td>RAEB</td>
<td>7</td>
<td>4.5</td>
<td>4.5/4.8</td>
<td>7/0/7</td>
<td>Complex anomaly*</td>
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<tr>
<td>109</td>
<td>M</td>
<td>61</td>
<td>RAEB</td>
<td>41</td>
<td>15.2</td>
<td>4.2</td>
<td>0/0/6</td>
<td>46,XY [20]</td>
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<tr>
<td>110</td>
<td>M</td>
<td>68</td>
<td>RAEB</td>
<td>33</td>
<td>17.5</td>
<td>4.6</td>
<td>4/1/5</td>
<td>46,XY,der(7)(t(1;7)(p10;q10),del(20)(q11) [20]</td>
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<tr>
<td>111</td>
<td>M</td>
<td>30</td>
<td>RAEBt</td>
<td>3</td>
<td>4.3</td>
<td>3.6</td>
<td>3/2/5</td>
<td>Complex anomaly*</td>
</tr>
<tr>
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<td>RAEBt</td>
<td>18</td>
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<td>1/4/5</td>
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<tr>
<td>113</td>
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<td>6/0/6</td>
<td>45,XY,del(5)(q12q13),i(8)(q10),–9,–21,21,–22,–r,–2,mar [8]</td>
</tr>
</tbody>
</table>

* Aberrations analyzed by DAPI images used for Q-FISH

**Assessment of TRF by Southern Blot.** Bone marrow aspirates were sedimented through Ficoll-Conray solution (Lymphoperal I; IBL, Fujikura, Japan), and mononuclear cells were obtained from the interface layers. Genomic DNA was extracted from frozen samples with use of a DNA extractor WB kit (Takara Biomedicals, Otsu, Japan). Terminal restriction fragment analysis was performed as reported previously (12, 14). TRF was defined as the peak intensity of telomere length and expressed in kb.

**Q-FISH Analysis of Metaphase Chromosomes.** Bone marrow aspirates and cell lines were cultured for 24 h without any mitogens before addition of colcemid (10 ng/ml) for 1 h. Cells were treated with 0.075 M KCl for 15 min at 37°C and fixed three times in methanol-acetic acid solution (3:1). Cell suspensions were dropped on slides and then dried overnight at room temperature. After the slides were fixed in 4% formaldehyde in PBS for 2 min, they were washed in PBS and treated with proteinase K (1 μg/ml) for 10 min at room temperature. We then added 10 μl of Cy3-labeled (CCCTAA)\(_3\) PNA probe mixtures (DAKO, Glostrup, Denmark) under a 20 × 20 mm coverslip, and the DNA was denatured for 3 min at 80°C. After hybridization for 2 h at 37°C, the slides were washed in PBS according to the manufacturer’s instructions (20). The slides were dehydrated with a cold ethanol series and air-dried; spread metaphases were then counterstained with DAPI. Metaphases were observed with an Olympus BX50 microscope (Olympus, Tokyo, Japan) with a charge-coupled device camera, and Cy-3 images were converted to arbitrary TFU with use of IPLab Spectrum (Scantyptic, Fairfax, VA). The mean TRF length of the K562 cell line of 3.3 kb, and that of the OM9;22 cell line was 4.4 kb; these TRF lengths were used as the control telomere lengths for every Q-FISH experiment. Individual telomere length was analyzed in at least five metaphases for each sample.
Statistical Analysis. The telomere length distributions were analyzed by Mann-Whitney t test using Statview 4.5 software (Macintosh version). P < 0.05 was considered significant.

RESULTS
Correlation between TFU and TRF Length in Healthy Volunteers and Cell Lines. In Fig. 1, panels a–e, the telomere fluorescence intensity in each metaphase is shown on the horizontal axis, and the sum of values of each signal intensity in each metaphase cell is shown on the vertical axis; these fluorescence signals in metaphase cells were superimposed and expressed as TFU in each subject (Fig. 1f). Telomere fluorescence signals in most metaphases obtained from a normal subject showed a single peak of fluorescence intensity from 1400 to 2500 TFU (Fig. 1, a–e). The sum of fluorescent intensity in a single subject (Fig. 1f) had a single peak, suggesting that the rate of reduction of telomeres among marrow cells in normal subjects is relatively homogeneous. These results indicate that telomere length differs among chromosomes within a metaphase but that marrow cells in normal subjects show homogeneous

Fig. 1 a–e, TFU histograms of each metaphase obtained from a healthy volunteer (case no. 205). f, histogram of TFU values from five metaphases. g, representative Q-FISH metaphase whose histogram is partially shown in d.

Fig. 2 Relationship between TFU detected by Q-FISH (vertical axis) and TRF length determined by Southern analysis (horizontal axis) obtained from healthy volunteers (○). ○ indicate K562 and OM9:22 cell lines. A significant correlation was detected: TFU = \(-1172 + 437\) (TRF); \(r^2 = 0.862\). × indicate TRF length and TFU values of MDS patients.
telomere shortening. This also indicates that the telomere fluorescence intensity obtained with the PNA probe in this study was stable and that the results of this study may be reproducible.

We then compared TFU values and TRF lengths in bone marrow aspirates obtained from healthy volunteers and cell lines. We found a significant correlation between TRF length and TFU value in healthy volunteers: $TFU = -1172 + 437(\text{TRF, in kb})$; $r^2 = 0.862$; Fig. 2; results of normal subjects are indicated by ●). The regression line for the TFU/TRF ratio in normal subjects demonstrated that 0 TFU corresponded to 2.68 kb (Fig. 2). On the basis of the linear correlation between TFU and TRF in normal subjects, we estimated that the telomere length distribution in individual metaphases in normal subjects ranged from 1.1 to 2.7 kb. Peak telomere length calculated by the formula was widely distributed in normal subjects, 6.7–9.1 kb (Fig. 2), whereas Southern analysis detected TRF lengths of 6.9–9.0 kb.

**Telomere Signals Detected by Q-FISH in MDS Patients.**

In contrast to normal subjects, telomere signals in MDS metaphases showed a very narrow range of fluorescence intensity at the single metaphase level. A representative metaphase obtained from a MDS patient (UPN 105) had a TFU value ranging from 650 to 980, corresponding to a range of 4.2–4.9 kb (Fig. 3a); the distribution of telomere fluorescence signals was quite narrow and weak, in contrast to that in normal subjects. Another metaphase in this MDS patient had a narrow range of telomere signals from 980 to 1200 TFU, corresponding to a range of 4.9–5.4 kb (Fig. 3e), and the sum of the TFU for five metaphases from this MDS patient again showed a narrow range of telomere signals, equivalent to 4.2–5.4 kb (Fig. 3f).

After we summed the fluorescence signals of metaphases analyzed in each patient, we plotted the range and peak TFU value in each MDS patient (Fig. 4). Most MDS patients had a narrow range and weak telomere signals compared with those in normal subjects. This suggests that MDS metaphases carried short telomeres with a very narrow distribution. We next compared the TRF lengths and peak TFU values in each MDS patient. As shown in Fig. 2 (MDS samples are indicated by ×), most MDS patients had low TFU values (telomeres of marrow metaphases) compared with the TRF length (telomeres of mononucleated marrow cells). The estimated TRF length of each MDS patient calculated by the formula obtained from normal

![Fig. 3 a–e. TFU histograms of each metaphase obtained from case 105. f. histogram of TFU values from five metaphases. g. representative Q-FISH metaphase whose histogram is shown in c.](image-url)
cases 201

The range of telomere fluorescence detected by Q-FISH in MDS patients is very narrow compared with that in normal subjects (Table 1). Any cases with normal karyotype by standard Q-banding had cytogenetically normal metaphases by Q-FISH. This study using Q-FISH demonstrated patterns of telomere signals in normal marrow metaphases; a single narrow metaphase from normal individuals showed relatively widely distributed intensity of telomere signals, indicating that the telomere length in each chromosome banding had cytogenetically normal metaphases by Q-FISH. Another notable finding is that each MDS patient had a very narrow distribution of intensities of telomere signals, and most of them had weak signals, i.e., short telomere lengths. Of note, there was a difference in TRF length detected by Southern blot analysis and peak TFU value detected by Q-FISH in each MDS patient. These metaphases measured by Q-FISH demonstrated the same cytogenetic abnormalities that were obtained by the conventional Q-banded technique noted in various cases (Table 1). Any cases with normal karyotype by standard Q-banding had cytogenetically normal metaphases by Q-FISH. Of the 13 MDS patients, 2 (UPN 107 and UPN 108) had two peaks of TFU values after the data were summed. Case 107 was diagnosed as RA with a normal male karyotype and TRF length of 9.7 kb. The patient demonstrated two peaks consisting of six metaphases (Fig. 5), and each metaphase showed a narrow spike. Three metaphases had a shorter spike (TFU value ranging from 400 to 500, corresponding to 3.7 kb), but the other three metaphases showed a second spike of TFU with higher fluorescence signals (700–900 TFU, corresponding to 4.5 kb; Fig. 5g). Case 108 was diagnosed as RAEB with complex chromosome anomalies and a short TRF of 4.5 kb. The patient also demonstrated two spikes of 4.5 and 4.8 kb calculated from TFU values, from metaphases by Q-FISH that had numerical cytogenetic anomalies as well as Q-banding (Table 1), and from the narrowly distributed fluorescence pattern (data not shown). The remaining 11 MDS patients had a single spike of TFU among metaphases.

DISCUSSION

Average telomere length has been shown to correlate with the proliferative potential of a cell line without telomerase (21). Similarly, the probability of all telomeres losing function increases as telomeres shorten (22). In MDS patients, it has been reported that average telomere length shortening appears to correlate with complex cytogenetic abnormalities, indicating genomic instability and a poor prognosis (12–15). Although average telomere length could be defined as the peak intensity of telomeric repeats, we obtained various widths of telomeric repeats by Southern blot analysis. This study using Q-FISH demonstrated patterns of telomere signals in normal marrow metaphases; a single narrow metaphase from normal individuals showed relatively widely distributed intensity of telomere signals, indicating that the telomere length in each chromosome was heterogeneous, in keeping with the previous observation by Martens et al. (23), who demonstrated that certain telomeres, including 17p, 22q, 19p, and 20q, in normal cells are consistently shorter than others. However, summing of telomere fluorescence intensities of normal marrow metaphases produced a single spike, suggesting a homogeneous component of dividing metaphases; thus, the peak of telomere signals of marrow cells (summation of signals) was linearly correlated with TRF length determined by Southern analysis.

In contrast, marrow cells in MDS patients showed a very narrow distribution of intensities of telomere signals, and most of them had weak signals, i.e., short telomere lengths. Of note, there was dissociation between TRF lengths detected by Southern analysis and peak TFU values determined by Q-FISH analysis. MDS patients examined in this study demonstrated variable TRF lengths ranging from 3.2 to 17.5 kb; however, peak TFU values were clustered at 400–900, corresponding to 3.6–4.8 kb (Fig. 2). This dissociation might be attributable to the difference in cell source examined rather than technical differences: Southern analysis used mononucleated whole marrow cells, whereas Q-FISH determined telomere signals in only marrow metaphases. Moreover, TFU signals in MDS metaphases were distributed in quite a narrow range, which is unlikely in normal marrow metaphases. Hence, TFU signals correspond to the telomere lengths of metaphases (cells in the mitotic phase of division); telomere lengths in MDS metaphases determined by Q-FISH were clustered in a narrow range, whereas whole marrow cells in MDS patients had variable telomere lengths detected by Southern analysis (Fig. 2). One possibility is that MDS marrow cells consist of at least two populations regarding telomere lengths, one derived from the MDS clone and the other from the normal background component, and that only MDS-derived metaphases were detectable by the Q-FISH method (Table 1). Although DAPI staining did not reveal any exact cytogenetic anomalies in the small number of metaphases used for Q-FISH, these findings suggest that the...
metaphases used for Q-FISH were certainly dysplastic cells and had short telomere lengths.

MDS is considered a hematopoietic stem cell disease; thus, the majority of MDS marrow cells, including metaphases, might originate from the MDS clone. In contrast, lymphocytes in MDS patients are generally believed to derive from the normal background. We could not find any cases with abnormal karyotypes by standard Q-banding but with cytogenetically normal metaphases by Q-FISH (Table 1), thus indicating that contamination of lymphocytes in MDS marrow samples may produce a difference in TRF length and TFU signals in MDS marrow cells (24).

It is reported that CD19+/ B lymphocytes carry longer telomeres (~1.5 kb) compared with T lymphocytes (23). Lymphocytes in the marrow usually do not enter the mitotic phase without any mitogens or stimuli; thus, the telomere lengths of lymphocytes cannot be assessed by the Q-FISH method.

In the present study, another important finding was that telomere signals in each MDS metaphase were clustered in a narrow range with short telomere length. In normal marrow cells, however, Q-FISH detected a wide distribution of long telomere lengths. In a recent study, the effect of telomere position was demonstrated in human cells (25). Hemann et al. (26) showed that the cellular response to telomere shortening depends on the shortest telomere within a cell rather than average telomere length. When an individual chromosome telomere becomes very short, it is recognized as DNA damage, and cell arrest or death occurs. If the rate of telomere reduction were homogeneous, the TFU signal distribution should be un-

Fig. 5 a–f, TFU histograms of each metaphase obtained from case 107. g, histogram of TFU values from six metaphases. h, representative Q-FISH metaphase whose histogram is shown in d.
changed and the intensity would decrease with a shift to the weak range. Cells with ultimate telomere shortening of certain chromosome(s) would then die. However, the Q-FISH pattern for MDS metaphases was quite different from that of normal marrow cells. This suggests that some MDS cells may survive and have the capability to divide with very short telomere lengths. Marten et al. (27) also showed that the distribution of telomere length becomes increasingly skewed during senescence of human cultured fibroblasts. In other words, the rate of telomere shortening among chromosomes in a MDS cell may be heterogeneous, and telomere shortening seems to cluster at the shortest telomere length of chromosomes.

It has been reported that overexpression of TRF-2 (a telomere binding protein) reduces the threshold of the critical telomere length at which cellular senescence occurs (28). Telomere-binding protein(s) may therefore have some role in the telomere dynamics in neoplastic cells as well as MDS cells; hence, MDS metaphases have ultimate telomere shortening with a narrow range of length. MDS cells with critical telomere shortening may die out by apoptosis. On the other hand, such cells may not enter the cell cycle, and Q-FISH would thus fail to detect telomere signals. This hypothesis further allows us to speculate that some MDS cells may cluster telomere lengths at the shortest length via certain factor(s), including telomerase, and then escape the checkpoint mechanism and survive. Nevertheless, the nature of the chromosome-specific regulation of telomere length has not been clearly demonstrated. It is postulated that a relationship exists between telomere shortening and the cell cycle (28, 29). The most plausible explanation is that critical telomere shortening of a single chromosome may trigger a p53-dependent DNA damage checkpoint that induces cellular senescence (30, 31). In MDS cells, however, telomere signals accumulate at short lengths.

Although we could not find any association between telomere shortening and cytogenetic abnormalities that related to telomere instability, MDS cells with ultimate telomere shortening may undergo cellular senescence, i.e., apoptosis. It has been demonstrated that telomeres in yeast maintain their length without telomerase by recombination (32). Because most MDS patients do not express a high level of telomerase activity and recombination-based telomere maintenance could be limited in human cells by the availability of long telomeres for elongation of short telomeres (27), the hematological characteristics in MDS that show dyshematopoiesis with maturation disturbance of hematopoietic cells may be closely associated with the telomere dynamics of this disease. Because most MDS patients show a high frequency of marrow cells exhibiting apoptosis, resulting in pancytopenia in the peripheral blood, ultimate telomere shortening might relate to these hematological features, and the escape of some MDS cells from the checkpoint could be a subject for analysis. Further study regarding telomere dynamics in nondividing but MDS-originated cells, if present, is required.

REFERENCES


21. Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. Telomere length...
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