Telomere Stability Is Frequently Impaired in High-Risk Groups of Patients with Myelodysplastic Syndromes

Junko H. Ohyashiki, Hiroshi Iwama, Naoyuki Yahata, Keiko Ando, Shigefumi Hayashi, Jerry W. Shay, and Kazuma Ohyashiki
First Department of Internal Medicine, Tokyo Medical College, Tokyo 160-0023, Japan [J. H. O., H. I., N. Y., K. A., S. H., K. O.], and Department of Cell Biology and Neuroscience, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9039 [J. W. S.]

ABSTRACT
Genomic instability induces an accumulation of genetic changes and may play a role in the pathogenesis of myelodysplastic syndromes (MDS). To clarify the possible association between genomic instability and clinical outcome in MDS patients, we compared telomere dynamics to the recently established International Prognostic Scoring System (IPSS) risk groups for MDS. We measured the terminal restriction fragments (TRFs) of 93 patients with MDS at the time of diagnosis, and telomerase activity was analyzed in 62 patients with MDS using the PCR-based telomeric repeat amplification protocol (TRAP) assay. A total of 53 of 93 MDS patients had TRFs within the age-matched normal range, and the remaining patients showed shortened TRFs (35 patients) or elongated TRFs (5 patients). MDS patients with shortened TRFs had a significantly low hemoglobin concentration (P = 0.04), a high percentage of marrow blasts (P = 0.02), and a high incidence of cytogenetic abnormalities (P < 0.05). The incidence of leukemic transformation was significantly high in patients with shortened TRF length (P < 0.05). In addition, patients with shortened TRF length were frequently seen in the IPSS high-risk group (P < 0.01). Most of the MDS patients had normal-to-low levels of telomerase activity, suggesting that changes in TRF length rather than telomerase activity may more accurately reflect the pathophysiology of MDS. MDS patients with shortened TRF length had a very poor prognosis (P < 0.01), suggesting that telomere dynamics may be linked to clinical outcome in MDS patients. Thus, an abnormal mechanism of telomere maintenance in subgroups of MDS patients may be an early indication of genomic instability. This study demonstrates that telomere stability is frequently impaired in a high-risk group of MDS patients and suggests that, in combination with the IPSS classification system, measurement of TRFs may be useful in the future to stratify MDS patients according to risk and manage the care of MDS patients.

INTRODUCTION
The MDS encompasses a heterogeneous group of disorders characterized by cytopenias and dysplastic features in the BM. Approximately 30% of patients with MDS show progression into AML (post-myelodysplasia AML; Ref. 1). After the initial FAB group classification in 1982, several risk classification systems for MDS have been proposed to determine both overall survival and which subgroups are most likely to develop AML (2, 3). Recently, an IPSS for MDS, based on the BM blast percentage, specific cytopenias, and cytogenetic patterns, has been proposed to develop a consensus prognostic risk-based analysis. In the IPSS, cytogenetic classification is one of the important elements used to determine clinical outcome (4).

Telomeres, the ends of chromosomes, consist of simple tandem repeats. In humans, it is reported that about 10–15 kb of (TTAGGG) repeats are found at the ends of all chromosomes (5). Telomeres progressively shorten with age in all somatic cells including blood cells and fibroblasts (6, 7). This reduction of telomere length is also present in various human cancer cells (8), although the biological significance of this reduction in telomere length is uncertain. It is thought that telomere shortening resulting from incomplete DNA replication may be related to genomic instability and an increased frequency of chromosome abnormalities leading to other mutations and disease progression. Telomerase is a ribonucleoprotein enzyme responsible for the complete replication of telomeres in the germ line (9). In addition, telomerase activity is detected in over 85% of primary human malignancies (10). There is mounting evidence that although telomerase may not be essential for the initial formation of tumor cell growth, progression and long-term growth of malignant human tumors in general are associated with activated telomerase (10, 11). Telomerase activity is not detected in most somatic cells, except for some cells of specific renewal tissues, such as hematopoietic stem cells (6, 12, 13).
However, the presence of telomerase does not necessarily imply stable and unchanging telomere lengths. Thus, there may be levels of functionally active telomerase that slow but do not prevent telomere shortening. Although it is not known why telomeres in telomerase-competent hematopoietic cells of renewal tissues get progressively shorter with age, one explanation may be the need to limit the proliferative capacity of hematopoietic cells as an anticancer mechanism. For example, in a recent study by Notaro et al. (14) of 11 fully engrafted BM recipients, the telomere length was significantly reduced in 10 of 11 recipients. This suggests that there was an increased replicative proliferation demand on stem cells at the time of transplant and during the engraftment phase (15). If telomere shortening is indeed an important determinant of aging in the hematopoietic system, then this may help explain the increased incidence of cancer in long-term survivors of BM transplant (16–18). Whereas this is only a correlation at present, it does suggest that an understanding of telomere dynamics in a variety of hematological disorders may provide fundamental insights into the molecular mechanisms involved.

MATERIALS AND METHODS

Patients. We examined TRFs in 109 samples from 93 consecutive patients with MDS (age, 25–85 years): (a) 50 patients with RA; (b) 3 patients with RAEB; (c) 20 patients with RAEB; (d) 12 patients with RAEB; and (e) 8 patients with chronic CMML. Telomerase activity was examined in 78 samples from 62 consecutive patients with MDS (age, 25–83 years): (a) 42 patients with RA; (b) 1 patient with RARS; (c) 10 patients with RAEB; (d) 6 patients with RAEB; and (e) 3 patients with CMML. In most (74 samples for TRF assay and 50 samples for telomerase assay) of the MDS patients, BM mononuclear cells were obtained at the time of diagnosis, and PB mononuclear cells were substituted only when BM cells were not available. PB mononuclear cells obtained from 78 healthy volunteers (age, 4–90 years) as well as BM mononuclear cells obtained from 12 healthy volunteers (age, 30–72 years) were used as controls for TRFs and telomerase activity. All samples were acquired after informed consent was obtained from the patient. Immortalized human leukemia cell lines HL60, U937, HAL-01, and OM9:22 were also analyzed as positive controls of telomerase activity. Cyto genetic analyses were routinely performed after short-term (<48 h) culture (19). For cytogenetic categorization, patients were divided into groups of those with normal karyotypes or those with single, double, or complex (i.e., ≥3 chromosomal anomalies) abnormalities.

Telomeric Repeat Analysis. TRF analysis was done as reported previously (19). Briefly, 10 µg of Hinfl-digested DNA of each sample was size-fractionated by electrophoresis on 0.8% horizontal agarose gels. After electrophoresis, Southern hybridization was carried out using a 32P-labeled (TTAGGG)4 telomeric probe. The range of telomere repeats of the autoradiograms was captured on an Image Master (Pharmacia Biotech, Uppsala, Sweden), and the telomere lengths were assessed quantitatively (19). We then defined the average TRF in each sample as the peak intensity of the telomere length, in kb (19). We also performed Mbol, RsaI, or TaqI digestion in some individuals to identify whether TRF length may be influenced by the methylation pattern of the first Hinfl site in the subtelomeric region.

Telomerase Assay. Telomerase activity was assessed according to the method of Kim et al. (10) and Piatyszek et al. (20) with modifications using an ITAS and an automated laser fluorescence DNA sequencer (21, 22). We prepared lysates from DMSO-free mononuclear cells from PB or BM cells stored at –80°C. Briefly, 50 µl of TRAP reactions including 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid extract, 10 pmol of fluorescent-labeled TS forward primer (5’-AATCCGTGCAGCAGGT-3’), 1 µg of T4 gene 32 protein (Boehringer Mannheim), and 2 units of Taq DNA polymerase (Takara Shuzou, Shiga, Japan) were placed over lyophilized fluorescent-labeled CX reverse primer (10 pmol; 5’-CCCTAACCCTTACCCTTACCCTAA-3’) using a wax barrier (Perkin-Elmer, Norwalk, CT). The microtube was incubated for 20 min at room temperature to allow for telomerase-mediated extension of the TS primer. The mixture then was heated at 90°C for 90 s and amplified using PCR (30 rounds at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min). For standardization of telomerase activity, we used 10 ag/assay of ITAS, which did not interfere with the TRAP assay (21, 22). The PCR products were subjected to 8% acrylamide denaturing electrophoresis in a ALF DNA sequencer II (Pharmacia Biotech) and analyzed by the Fragment Manager program (Pharmacia Biotech; Refs. 21 and 22). To compare the relative amount of telomerase activity between samples, the telomerase activity signals were normalized to the signal from 10 ag/assay of ITAS and then expressed as a relative value of ITAS. Experiments were carried out at least twice to improve the reliability of the results. We calculated the mean relative values of telomerase activity in each sample.

Statistical Analysis. We used the Kaplan-Meier method to calculate survival curves. We also used the χ2 test when appropriate. Hematological data are expressed as the mean ± SD. Comparisons between groups were analyzed using the Mann-Whitney U test. Values of P < 0.05 were considered significant. The statistical tests were performed using the Statview (Brain Power Inc., Calabases, CA) software package for the Macintosh personal computer.

RESULTS

Telomere Length and Telomerase Activity in MDS Patients Compared to Normal Individuals. We compared TRFs (a measurement of telomere length) and telomerase activity in PB and BM cells obtained from age-matched healthy volunteers to those of MDS patients (Fig. 1). The present results and those obtained previously (6, 19) from healthy volunteers show that there were no differences between TRFs and telomerase activity in PB cells and those in BM cells (22). Whereas TRFs in normal controls varied (6), overall TRFs were shorter with increased age (P < 0.05; Fig. 1A). In MDS patients, there was no obvious relationship between TRF length and age (Fig. 1B). In 53 of 93 MDS patients, TRFs were within the age-matched normal range. The remaining 40 patients had shortened TRFs (35 patients) or elongated TRFs (5 patients). Samples showing elongated TRFs were also digested with Mbol, RsaI, or TaqI; however, their TRFs were approximately the same size as
those digested with **Hinfl**, indicating that elongated TRFs were not due to differences in methylation.

Using an extract derived from $2 \times 10^3$ cell equivalents/assay, relative telomerase activity is generally less than 1.0 in samples obtained from healthy volunteers who are more than 40 years old (Fig. 2A). Based on the presence of telomerase activity in PB and BM cells obtained from healthy volunteers (6), we tentatively defined the level of telomerase activity as reported previously: (a) normal, lower or equal to that expected relative to age (i.e., <mean + SD); low, above normal and up to 10 relative value; moderate, 10–50 relative value; high, >50 relative value (22). As shown in Fig. 2, most of the MDS patients had normal-to-low levels of telomerase activity. Three patients (one patient with RA and two patients with RAEBt) had high levels of telomerase activity. The two RAEBt patients with high telomerase activity developed to acute leukemia shortly after the initial diagnosis. The remaining RA patient with high telomerase activity developed acute leukemia 3 years after the initial diagnosis. Because patients with high telomerase activity were uncommon in MDS, we conducted a statistical analysis on telomere length rather than telomerase activity.

**Telomere Length, FAB Subtypes, and Cytogenetic Patterns.** Among 93 patients with MDS, the peak TRF lengths (mean ± SD) were 8.1 ± 2.0 kb in RA patients, 7.7 ± 2.6 kb in RARS patients, 8.6 ± 3.6 kb in RAEB patients, 7.6 ± 2.8 kb in RAEBt patients, and 8.0 ± 3.0 kb in CMML patients; no significant difference in mean TRF length was noted among each FAB subtype. Because the number of patients with elongated TRFs was too small to perform a statistical analysis, we excluded MDS patients with elongated TRFs from the statistical analysis as mentioned below. Patients with shortened TRF length were seen in RA/RARS (18 of 53 patients; 34%) as well as in RAEB/RAEBt (15 of 32 patients; 47%), indicating that shortened TRF is not simply related to the subtypes of FAB classification (no significance as calculated by the $\chi^2$ test). A significantly high incidence of abnormal karyotypes was noted in patients with shortened TRFs; of the 88 MDS patients [excluding those having elongated TRFs ($n = 5$)], 23 of 35 patients with shortened TRF lengths and 18 of 53 patients with normal TRF lengths were shown to have abnormal karyotypes ($P < 0.01$).

Based on the difference in TRF lengths in MDS patients, we separated MDS patients into two groups according to TRF lengths as described in Fig. 1. The horizontal axis indicates the age of subjects in years, and the vertical axis indicates peak TRF length in kb.

**Fig. 1** Comparison of peak telomere length between normal individuals (A) and MDS patients (B). The horizontal axis indicates the age of subjects in years, and the vertical axis indicates peak TRF length in kb.

**Fig. 2** Comparison of relative telomerase activity between normal individuals (A) and MDS patients (B). The telomerase activity in each subject is expressed as the mean relative value compared to an ITAS (vertical axis). The horizontal axis indicates the age of subjects in years.

To understand the relationship between telomere length and telomerase activity, we compared the two groups of patients. The results showed that patients with normal or low telomerase activity had longer telomeres than those with high telomerase activity. This indicates that telomerase activity may play a role in the maintenance of telomere length in MDS patients.
length and compared clinical and hematological features (Table 1). MDS patients with shortened TRFs had a significantly low hemoglobin concentration (P = 0.04) and a high marrow blasts percentage (P = 0.02). Assessment of the risk factor element based on the IPSS showed that MDS patients with short TRFs had a significantly high marrow blast percentage (P < 0.05), multiple cytopenia (P < 0.05), and poor cytogenetic changes (P < 0.05; Table 1).

**Relationship between Telomere Length and Leukemic Transformation.** We next compared the incidence of leukemic transformation between patients with normal-range TRF and those with abnormal TRF length. A total of 54% (19 of 35) of patients with shortened TRF developed AML, whereas only 28% (15 of 53) of those with normal TRF developed AML. The incidence of leukemic transformation was significantly higher in MDS patients with shortened TRF length compared to those with normal-range TRFs (P < 0.05). For each FAB subtype, a statistically significant incidence of leukemic transformation was found only in RAEB with a shortened TRF length (P < 0.05). For each FAB subtype, no significant difference in the incidence of leukemic transformation was found in patients with chromosomal abnormalities (one chromosomal change V, two chromosomal abnormalities V, three or more chromosomal changes).

To determine whether changes of TRF length were associated with disease progression in MDS, we also examined TRF and telomerase activity before and after disease progression in 16 patients. TRF length did not change in 13 of 16 patients, whereas a further reduction of TRF length was noted in the remaining 3 patients. In the 16 patients examined, the level of telomerase activity did not change, even when TRFs were reduced after disease progression.

**Telomere Length and IPSS Risk Factor Analysis.** Telomere lengths among IPSS risk groups are shown in Table 2. Among 93 patients with MDS, 4 patients with CMML were excluded from IPSS analysis because of high leukocyte counts (proliferative type). One patient with RA was also excluded from the statistical analysis because the hematological data were incomplete. A total of 88 MDS patients were separated into the following groups, according to the recently established IPSS guidelines: (a) 14 patients in the low-risk group; (b) 47 patients in the intermediate-1 risk group; (c) 14 patients in the intermediate-2 risk group; and (d) 13 patients in the high-risk group. The peak TRF length was significantly shorter in the high-risk group compared to that in the low-risk group (P = 0.03) or the intermediate-1 risk group (P = 0.007) as calculated by the Mann-Whitney U test. In addition, the peak TRF lengths were significantly shorter in the intermediate-2 risk group compared to those in the low-risk group (P = 0.02) or the intermediate-1 risk group (P = 0.03). Patients with shortened TRF length were frequently seen in the intermediate-2 risk group (9 of 14 patients; 64.3%) and the high-risk group (9 of 12 patients (75%) in the high-risk group had shortened TRFs (Table 2). This association between telomere length and IPSS risk analysis was statistically significant (P < 0.01; $\chi^2 = 15.599$).

**Survival Probability in MDS Patients Depends on TRF Length.** The MDS patients were classified into three groups according to telomere length: (a) normal TRFs; (b) shortened TRFs; and (c) elongated TRFs. MDS patients with normal TRFs had a favorable prognosis (P < 0.01) compared to those with abnormal TRF length (Fig. 3).

**DISCUSSION**

MDS is characterized by an ineffective hematopoiesis resulting from rapid cell division. In the present study, the average telomere length, expressed as the peak TRF length, varied in...
each MDS patient. However, telomere stability was impaired in a subset of MDS patients. Unlike dysplastic lesions of solid tumors (23), a high level of telomerase activity was not common in MDS patients. Using the in situ telomerase activity technique (24), we could not find telomerase-positive cells in most of the MDS patients, with the exception of a patient with RAEBt whose BM had a small population of cells expressing telomerase signals (data not shown). Therefore, an accelerated telomere erosion due to rapid cell division may not be restored in most of the MDS cells because telomerase activity is insufficient to maintain telomere length in those patients. Loss of telomere stability may induce further genetic changes such as loss of heterozygosity, gene amplification, or chromosomal rearrangement (8). Unstable telomeres without elevated telomerase activity may be a biological property of MDS cells, and this phenomenon is probably important in the pathophysiology of the disease. Recently, several investigators have demonstrated that BM hematopoietic cells of MDS patients have increased apoptosis compared to normal BM cells (25, 26). Taken together with our results, the biological characteristics of MDS cells are different from that of de novo acute leukemia cells. Therefore, telomere length rather than telomerase activity may play an important role in the pathogenesis of MDS.

We and others have previously shown that telomere shortening in MDS patients is frequently associated with complex cytogenetic changes (21, 27). In the current study, we found that changes in telomere length were closely associated with the presence or absence of detectable chromosome abnormalities rather than patterns of chromosome change. We also found that MDS patients with shortened TRFs had a significantly high incidence of leukemic transformation and a poor prognosis. Our previous study of microsatellite alterations demonstrated that genetic instability at multiple microsatellite loci often occurs before leukemic transformation (28). Similarly, impairment of telomere stability may also occur as an early genetic event in a certain subset of MDS patients. Therefore, we proposed that shortened TRF length at the time of MDS diagnosis may be a new parameter for helping to stratify MDS patients according to risk.

The IPSS new guidelines define critical prognostic features of MDS patients and provide improved prognostic evaluation compared with FAB subtypes. We found a close association between TRF length and IPSS risk group analysis: the mean TRF length was significantly shorter in patients of the intermediate-2 and high-risk groups compared to those in patients of the low-risk and intermediate-1 risk groups. Our study clearly shows that the molecular background of patients in the intermediate-2 and high-risk groups is different from that of patients in the intermediate-1 and low-risk groups. Therefore, telomere length in combination with IPSS may be an independent molecular determinant for evaluating prognosis in MDS.

The IPSS new guidelines define critical prognostic features of MDS patients and provide improved prognostic evaluation compared with FAB subtypes. We found a close association between TRF length and IPSS risk group analysis: the mean TRF length was significantly shorter in patients of the intermediate-2 and high-risk groups compared to those in patients of the low-risk and intermediate-1 risk groups. Our study clearly shows that the molecular background of patients in the intermediate-2 and high-risk groups is different from that of patients in the intermediate-1 and low-risk groups. Therefore, telomere length in combination with IPSS may be an independent molecular determinant for evaluating prognosis in MDS.

Table 2 Telomere length and IPSS risk group analysis

<table>
<thead>
<tr>
<th>IPSS groups</th>
<th>No. of patients with normal TRFs</th>
<th>No. of patients with shortened TRFs</th>
<th>No. of patients with elongated TRFs</th>
<th>TRF (mean ± SD)</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI*</td>
<td>12/14 (85.7%)</td>
<td>2/14 (14.3%)</td>
<td>0/14</td>
<td>8.5 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>Intermediate-1</td>
<td>33/47 (70.2%)</td>
<td>11/47 (23.4%)</td>
<td>3/47 (6.4%)</td>
<td>8.6 ± 2.7</td>
<td>P1</td>
</tr>
<tr>
<td>CI</td>
<td>55.1–82.7%</td>
<td>35.1–87.2%</td>
<td>17.3–44.9%</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Intermediate-2</td>
<td>5/14 (35.7%)</td>
<td>9/14 (64.3%)</td>
<td>0/14</td>
<td>6.8 ± 2.2</td>
<td>P2</td>
</tr>
<tr>
<td>CI</td>
<td>12.8–64.9%</td>
<td>55.1–87.2%</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>High</td>
<td>3/13 (23.1%)</td>
<td>9/13 (69.2%)</td>
<td>1/13 (7.7%)</td>
<td>6.4 ± 2.0</td>
<td>P3</td>
</tr>
<tr>
<td>CI</td>
<td>5.5–57.2%</td>
<td>42.8–94.5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53/88 (60.1%)</td>
<td>30/88 (34.5%)</td>
<td>4/88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference of TRF length was calculated by the Mann-Whitney U test; NS, not significant; P1 = 0.0279; P2 = 0.0159; P3 = 0.0288.

b CI, confidence interval.

Fig. 3 Survival probability of MDS patients separated by peak telomere length. Normal TRF indicates patients within the age-matched normal telomere length (mean ± 2 × SD). Abnormal TRF indicates patients with shortened telomeres and elongated TRF compared to the age-matched telomere length (<mean – 2 × SD). A significant difference in survival probability was noted between MDS patients with abnormal TRF and those with normal TRF (P < 0.05).
could occur as a late genetic event in a subset of MDS, as reported in a blastic crisis of chronic myeloid leukemia (29, 30). We also found one RA patient with high telomerase activity who subsequently developed acute leukemia 3 years after initial diagnosis. This patient had elongated telomeres during the course of disease. Whereas the biological and clinical significance of elongated telomeres is still uncertain, we cannot exclude the possibility of an abnormal mechanism of telomere maintenance in such patients. At the present time, we cannot draw a final conclusion about the clinical significance of telomerase activity in MDS patients because there were so few patients with high telomerase activity in this study. In conclusion, telomere stability may be closely related to the pathophysiology of MDS. Therefore, an analysis of telomere dynamics may be useful for risk classification systems in combination with IPSS.

REFERENCES

1. Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A. G., Graithauf, H. R., and Sultan, C. Proposals for the classification of the pathophysiology of MDS. Therefore, an analysis of telomere study. In conclusion, telomere stability may be closely related to abnormal mechanism of telomere maintenance in such patients. At the present time, we cannot draw a final conclusion about the clinical significance of telomerase activity in MDS patients because there were so few patients with high telomerase activity in this study. In conclusion, telomere stability may be closely related to the pathophysiology of MDS. Therefore, an analysis of telomere dynamics may be useful for risk classification systems in combination with IPSS.
Telomere Stability Is Frequently Impaired in High-Risk Groups of Patients with Myelodysplastic Syndromes
