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Research Paper

Increase of telomerase activity and hTERT expression in myelodysplastic syndromes

Federica Briatore,[1,†] Giuseppina Barrera,[1,*] Stefania Pizzimenti,[1] Cristina Toaldo,[1] Chiara Della Casa,[2] Stefano Laurora,[1] Piergiorgio Pettazzoni,[1] Mario Umberto Dianzani[1] and Dario Ferrero[2]

[1]Dipartimento di Medicina e Oncologia Sperimentale; Sezione di Patologia Generale; [2]Dipartimento di Medicina e Oncologia Sperimentale; Sezione di Ematologia (Divisione di Ematologia dell'Università di Torino); Università di Torino; Torino, Italy

†These authors contributed equally to this work.

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Telomerase enzyme, containing a catalytic subunit, the human telomerase reverse transcriptase (hTERT), and a small integral RNA component, synthesises the telomeres, the ends of eukaryotic chromosomes. Inhibition of telomerase activity leads the cells to senescence and death. Myelodysplastic syndromes (MSD) are hematological malignancies characterized by peripheral blood cytopenia and ineffective hematopoiesis. Telomerase activity and hTERT expression in MDS patients were independently investigated by different groups obtaining contradictory results. We analyzed telomerase activity and hTERT expression in the bone marrow of ten control, 15 MDS patients and two patients with AML, likely evolved from a previous MDS. Moreover, the expression of c-myc, mad1, p53 (transcription factors involved in hTERT expression regulation), has been investigated. Telomerase activity and hTERT expression increased in the MDS patients with respect to the controls. The analysis of the MDS subgroups, indicated that patients with more severe disease demonstrated significantly higher levels of hTERT expression and telomerase activity with respect to the patients with more favorable disease. c-Myc and p53 expressions were not significantly different between controls and MDS patients, whereas mad1 expression was increased in MDS patiens, particularly in those with more favorable disease. We hypothesize that mad1 increase can contribute to reduce the hTERT expression in the early stage of disease and we suggest that hTERT expression and telomerase activity, whether confirmed in larger series of cases could support other parameters in the diagnosis and stadiation of MDS.

*Correspondence to: Giuseppina Barrera; Dipartimento di Medicina e Oncologia Sperimentale; Università di Torino; Corso Raffaello 30; Torino 10135 Italy; Tel.: +39.011.6707772; Fax: +39.011.6707753; Email: giuseppina.barrera@unito.it

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Introduction

Telomerase is a specialized enzyme that synthesizes telomeres.[1] Telomeres correspond to the ends of eukaryotic chromosomes and are specialised structures containing unique (TTAGGG)n repeats, protecting the chromosomes from DNA degradation, end to end fusions, rearrangements and chromosome loss.[1] The number of telomere repeats decreases (by 50–200 nucleotides/cell division) during aging of normal somatic cells[2] and it has been proposed to be a mitotic clock marking progression of a cell toward the end of its replicative lifespan. Synthesis and maintenance of telomeric repeats are mediated by telomerase, a ribonucleoprotein complex, which contains a catalytic subunit, the human telomerase reverse transcriptase (hTERT) and a small integral RNA component (hTR), utilised as a template for the synthesis of the dGT-rich strand of telomeres.[1] Inhibition of telomerase activity leads the cells to senescence[3] and death. Although most normal adult somatic cells do not express telomerase, immortalized cells such as tumor cells express this enzyme.[4,5]

Telomerase activity and hTERT expression level in AML has been investigated in different works.[6-8] Recently, Huh et al.[9] demonstrated by real-time RT-PCR that hTERT expression was higher in patients with AML at diagnosis and during relapse than during remission and that patients who failed to achieve complete remission had a high level of hTERT mRNA, this suggesting that hTERT mRNA may be a useful marker for prognosis prediction and disease monitoring in AML patients. Pharmacological telomere inhibition can sensitise drug-resistant and drug-sensitive cells to chemotherapy.[10] Telomerase, therefore, has been proposed to represent a novel and potentially selective target for cancer therapy.

Myelodysplastic syndromes (MSD) represent hematological malignancies that typically affect old people.[11] They arise as a consequence of genetic mutations (more frequently chromosome deletions or duplications) in a pluripotent hematopoietic stem cell, that acquires a survival/proliferative advantage over normal ones and a more or less pronounced differentiation impairment.[11,12] MDS patients display peripheral blood cytopenia (usually anemia ±

thrombocytopenia and/or neutropenia) in spite of a bone marrow (BM) cellularity that is often at an increased level (ineffective hematopoiesis). Various degrees of impaired maturation, increased apoptosis and immature (blast) cell accumulation characterize BM cellularity in MDS patients.[13] Clinical features are dominated by cytopenia-related complications and by evolution into acute myeloid leukemia, that occurs in a variable proportion of cases (5–50%),[14] depending on some particular MDS features (in particular, the type of cytogenetic abnormalities and BM blast percentage).[15] It has been suggested that telomerase activity and hTERT expression may be prognostically important in patients with MDS.[16,17] However, a contemporary analysis of telomerase activity and hTERT expression in MSD patients has not yet been performed. Regulation of hTERT expression depends on different transcription factors that bind hTERT promoter[18] and positively or negatively control hTERT expression. In particular the c-Myc, Max and Mad1 (Max dimerization protein 1) network can regulate hTERT expression through the binding to E-box binding sites.[19] Moreover, hTERT expression may be negatively regulated by p53 via the p21/E2F1 pathway.[20]

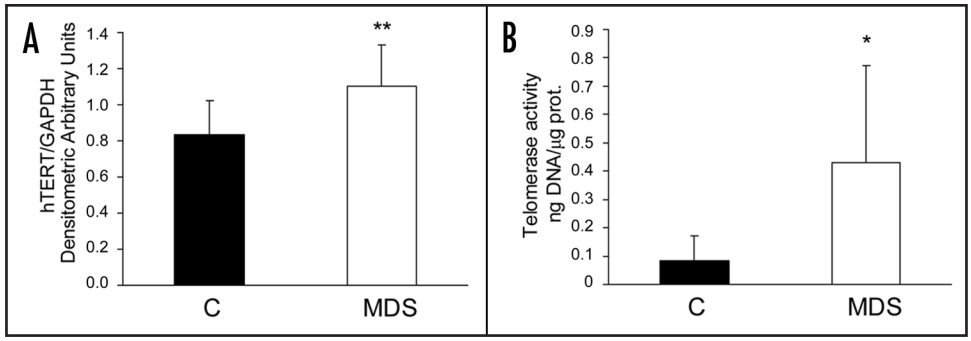

Figure 1. hTERT expression and telomerase activity. (A) hTERT mRNA levels determined by RT-PCR in the bone marrow of controls (C) and MDS patients. Relative quantification of RT-PCR products was performed by densitometric scanning. Data were normalized using the GAPDH signal and are expressed as means ± SD of arbitrary densitometric units. (B) Telomerase activity. The activity was evaluated in the bone marrow of controls (C) and MDS patients and it was calculated in term of ng DNA produced/µg protein. Data are expressed as means ± SD. Student's t-test: *p < 0.05, **p < 0.01.

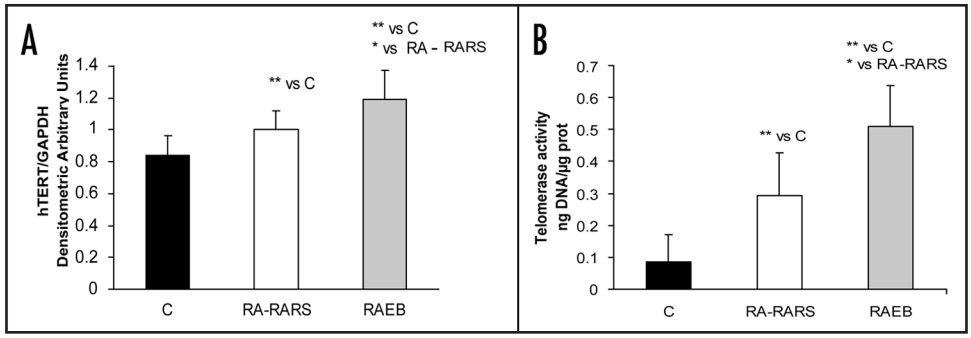

Figure 2. hTERT expression and telomerase activity in MDS subgroups. (A) hTERT mRNA levels determined by RT-PCR in the bone marrow of controls (C), RA-RARS and RCMD-RAEB patients. Relative quantification of RT-PCR products was performed by densitometric scanning. Data were normalized using the GAPDH signal and are expressed as means ± SD of arbitrary densitometric units. (B) Telomerase activity. The activity was evaluated in the bone marrow of controls (C) RA-RARS and RCMD-RAEB patients and it was calculate in term of ng DNA produced/µg protein. Data are expressed as means ± SD. Variance analysis: *p < 0.05, **p < 0.01.

Microarray studies, in the BM of MDS patients, demonstrated that mad1 is frequently abnormally expressed.[21] Moreover, an upregulation of c-myc expression has been demonstrated in MDS with trisomy 8.[22] Thus, an alteration of hTERT transcription factors may contribute to hTERT expression disregulation in MDS patients. For this reason, we investigated telomerase activity and hTERT expression, as well as c-myc, mad1 and p53 expression, in BM mononuclear cells of 17 MDS patients and of ten normal controls and evaluated the prognostical relevance of these parameters.

Results

hTERT expression and telomerase activity. hTERT expression is shown in Figure 1A. The mean of hTERT expression in the bone marrow of the controls was significantly lower ($p < 0.01$) than that found in the MDS patients. The values were quite homogeneous, and the datum was significant in spite of the fact that the increase of hTERT expression was only by 32% with respect to the control value. On the other hand, telomerase activity (Fig. 1B), evaluated in the bone marrow of the MDS patients was highly variable (as demonstrated by the standard deviation value), however the mean was significantly higher than that evaluated in the control donors ($p < 0.05$). Control BM exhibited a low level of telomerase activity and an intermediate hTERT expression. In addition, very high levels of telomerase activity was present in MDS specimens with intermediate and elevated hTERT expression. The analysis of the MDS subgroups, based on WHO classification, indicated that patients with more severe disease (intermediate or high International Prognostic Score System, IPSS) (RCMD and RAEB1 and 2) demonstrated significantly higher levels ($p < 0.05$) of hTERT expression (Fig. 2A) and telomerase activity (Fig. 2B) with respect to the patients with more favourable disease (low IPSS) (RA, RARS). However, no significant difference has been found on the basis of blast percentage. The two patients with AML derived from MSD had values of hTERT expression (arbitrary densitometric units that were 1.30 and 0.91, respectively) and telomerase activity (ng DNA/µg protein that were 0.40 and 0.30 respectively) near to the MDS medium value.

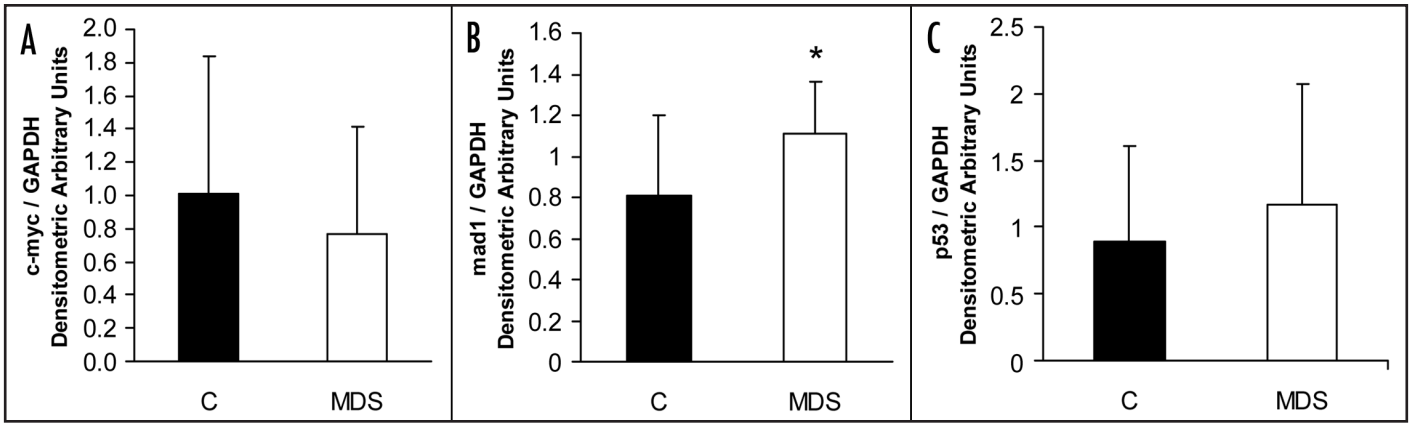

Figure 3. Expression of transcription factors. c-myc (A), mad1 (B) and p53 (C) mRNA levels determined by RT-PCR in the bone marrow of controls (C) and MDS patients. Relative quantification of RT-PCR products was performed by densitometric scanning. Data were normalized using the GAPDH signal and are expressed as means ± SD of arbitrary densitometric units. Student's t-test: *p < 0.05.

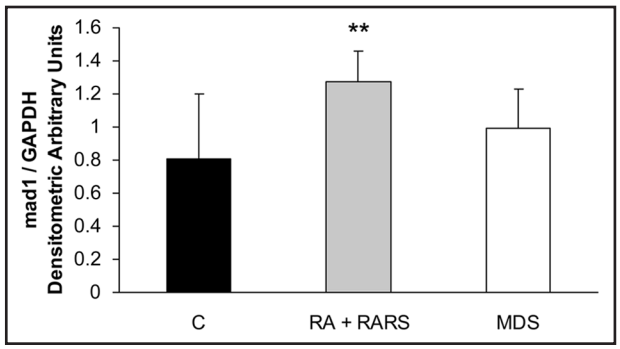

Figure 4. Mad1 expression. Mad1 mRNA levels determined by RT-PCR in the bone marrow of controls (C), RA-RARS and RCMD-RAEB patients. Relative quantification of RT-PCR products was performed by densitometric scanning. Data were normalized using the GAPDH signal and are expressed as means ± SD of arbitrary densitometric units. Variance analysis: **p < 0.01.

Telomerae activities and the expression levels of hTERT mRNA in both controls and MDS patients were well correlated. The Spearman rank correlation was 0.524 and p = 0.0486.

c-Myc, mad1 and p53 expression. In order to evaluate whether the expression of some important transcription factors involved in hTERT expression regulation can be modified in the BM of MDS patients with respect to the BM of the controls, we tested for c-myc, mad1 and p53 expressions. c-Myc and p53 gene expressions were not significantly different between the two groups, whereas mad1 expression was significantly increased ($p < 0.05$) in MDS patients with respect to the controls (Fig. 3). The analysis of the MDS subgroups, based on WHO classification, indicated that the value of mad1 expression in patients with low risk disease (RA, RARS) was quite significantly higher and highly significant ($p < 0.01$) with respect to the control value (Fig. 4). Conversely, the value of mad1 expression in RCMD and RAEB patients (excluding RA and RARS patients) was not significantly greater with respect to controls (Fig. 4). A significant inverse correlation exists between the expression levels of mad1 and hTERT in low risk patients (RA and RARS). The Spearman rank correlations in these groups was -0.598 and p = 0.0339.

Discussion

MDS are diseases characterized by genetic instability and ineffective hematopoiesis associated with the telomere shortening.[31] Telomere decline is detected in more than 60% of MDS patients, and no apparent change in telomere length has been observed despite higher telomerase activity in MDS.[32] Therefore, it has been suggested that telomerase activity becomes increased in MDS presumably when telomere loss generates selective pressure for cell immortality.[33] hTERT is differentially expressed in various haematologic stem cell disorders and contrasting data have been reported. Several reports indicate that in AML cells high telomerase activity correlates well with hTERT expression.[7,34] Conversely, conflicting results have been reported in chronic myeloid leukemia (CML) and in MDS: indeed, Bock et al.[17] reported that hTERT is expressed at higher levels in refractory anemia (RA) and CML compared to other haematopoietic stem cell disorders and non-neoplastic haematopoiesis. On the contrary Campbell et al.[35] recently reported that hTERT is downregulated in the hematopoietic stem cells of patients with chronic myeloid leukaemia. Oshima K, et al.[36] found that telomerase activity is higher in AML/MDS than in MDS and controls, whereas opposite results were reported by other authors[16] that did not find significant differences in telomerase activity in bone marrow of MDS patients compared to controls. It must be remarked that in literature data telomerase activity and hTERT expression were independently investigated by different groups in MDS patients, and this may account for some discrepancies in reported results.

In the present study we tested both hTERT expression and telomerase activity in the same group of MDS patients and found that telomerase activity as well as hTERT expression were higher in MDS patients and AML/MDS than in controls, the increase being more pronounced in patients with more severe disease (RCMD, RAEB, AML), in agreement with the results reported by Oshima et al.[36] However, the telomerase activity appeared to be

extremely variable in the different patients, and this observation may account for the different results reported by other authors[16] On the other hand, hTERT expression was found to be more homogeneously elevated in MDS patients, according to the results reported by Oshima et al.[36] who showed that the percentage of hTERT-BM positive cells was higher in AML-MDS and MDS than in controls.

Moreover, the increase in hTERT expression and telomerase activity in more severe MDS cases may suggest that these parameters may be used as a prognostic indicator, however this possibility should be evaluated in larger studies.

Unexpected results have been obtained in analyzing the expression of some transcription factors of the hTERT gene. C-myc gene, which positively regulates hTERT expression[37] was not increased, whereas mad1 gene, which negatively regulates hTERT expression,[38] was increased, particularly in low IPSS MDS. These data indicate that in MDS patients hTERT expression is probably modulated through different mechanisms or other transcription factors. It has been recently reported that the hTERT gene is a target for SMYD3, a histone methyltransferase implicated in oncogenesis[39] which directly trans-activated the hTERT gene. Moreover, the regulation of hTERT expression may depend on the indirect stimulation of hTERT promoter, as well as it occurs in HTLV-I infected cells, in which the Tax stimulates the hTERT promoter through the nuclear factor κ B pathway.[40] In addition to the regulation of hTERT expression, c-Myc, mad1 and p53 proteins have other important functions in cell growth regulation. The ratio Myc/Bcl-2 has been found to be increased in CD34 marrow cells of MDS patients compared to those of normal and AML individuals[41] and this datum has been related to the increase in apoptotic cells that mainly characterizes MDS without blast excess. Other authors found increased values of c-myc expression in trisomy 8 MDS patients[22] and correlated this datum with the resistance to apoptosis of trisomy 8 cells. In effect c-myc can have both a proapototic and a growth-inducing role[42] and the conclusive results about the role of its expression in MDS should be drawn from a very high number of patients divided in specific caryotype groups. The action of c-myc products can be antagonized by Mad family members[43] which act as transcription repressors. We found that mad1 gene expression was increased in the BM of MDS patients, in particular in those with low IPSS. Mad1 gene has been found abnormally expressed in MDS patients[44] and we can postulate that its increase may be related to the ineffective differentiation and the reduced proliferation of BM cells in early MDS patients. p53 gene expression has been investigated in MDS patients in order to clarify its role in apoptosis induction. Immunoistochemical analysis demonstrated that the number of cells which express p53 was higher in the BM of MDS patients in leukemic transformation, whereas in AML not derived from MDS, the frequency of p53-positive cells is lower.[45] On the other hand, other authors found that the apoptotic rate was independent from the expression of p53, bcl-2 and ras in AML and MDS.[46] Our results indicated that the expression of p53 in the BM of MDS patients was not significantly different from the controls, however we found a very high level of p53 expression in three of the patients with low IPPS and in one of the patients with intermediate IPPS. No patients with high IPPS showed elevated p53 expression in agreement with the reported low apoptosis level in high risk MDS.[47]

Taken together our results demonstrated that hTERT expression and telomerase activity are increased in BM of MDS patients compared to controls and this increment seems to correlate well with an intermediate/high risk according to the international prognostic score. Conversely, mad1 expression seems to be more increased in low risk patients and inversely correlated to hTERT expression. However, it must be emphasized that our results have been obtained from relatively few patients. Due to the great heterogeneity in clinical features, BM cellularity and genetic abnormalities among MDS patients, further studies on larger series of cases are required to confirm the possible prognostic role of hTERT expression and/or telomerase activity in MDS patients and their correlation to established prognostic features.

Materials and Methods

Patients. Bone marrow samples were taken from controls and MDS patients during diagnostic procedures, after consent had been obtained. Controls included seven untreated, early-stage non-Hodgkin's lymphoma patients, whose bone marrow taken for staging procedures had been found histologically and immunophenotypically normal, and three healthy donors. MDS cases included 15 patients with true MDS according to WHO classification,[23] and two with AML likely evolved from a previous undiagnosed MDS (AML with multilineage dysplasia and previous cytopenia for at least 6 mo before AML diagnosis).

Table 1 illustrates the main clinical and hematological features of MDS group patients. The group included 11 men and six women with a median age of 72 (42–80). Diagnosis, according to WHO classification, was "refractory anemia" (RA) in two, "refractory anemia with ring sideroblasts" (RARS) in four, "refractory cytopenia with multilineage dysplasia" (RCMD) in three, "refractory anemia with excess of blasts, type 1" (RAEB1) in two, "refractory anemia with excess of blasts, type 2" (RAEB2) in four, in one case secondary to previous essential thrombocythemia; and "acute myeloid leukemia with multilineage dysplasia" (AML) in two. Cytogenetic analysis was successfully performed in 16/17 patients: a normal caryotype was found in ten patients, including 8/9 patients without blast excess (RA, RARS, RCMD), 1/2 RAEB1, 1/4 RAEB2, 1/2 AML, whereas one or more cytogenetic abnormalities were found in six patients (1 RCMD, 1 RAEB1, 3 RAEB2, 1 AML). Prognostic score (IPSS),[15] evaluated in 14 MDS patients, was "low risk" in five, "intermediate 1 risk" in three, "intermediate 2 risk" in three and "high risk" in three patients, respectively.

Fourteen patients were studied before receiving any specific therapy, whereas four had been previously treated with recombinant erythropoietin (epo).

RNA isolation and semi-quantitative RT-PCR analysis. Low density bone marrow cells were separated on a ficoll-hypaque (Lymphoprep) gradient at 1077 g/l. Total RNA was isolated using the RNA fast Kit (Molecular System, Genenco, Milano,

Table 1 **Main clinical features of MDS group patients**

Patient #	Sex	Age	Diagnosis*	Caryotype	IPSS°	Previous treatment
1	F	79	RA	46,XX	low	no
2	M	72	RA	46,XY	low	no
3	F	72	RARS	46,XX	low	no
4	M	42	RARS	46,XY	low	epo
5	M	73	RARS	46,XY	low	no
6	M	80	RARS	N.D.	N.D.	no
7	M	74	RCMD	46,XY	intermed. 1	no
8	M	65	RCMD	46,XY	intermed. 1	no
9	F	75	RCMD	46,XX;del5q;del7q	intermed. 2	epo
10	M	64	RAEB 1	46,XY	intermed. 1	epo
11	M	55	RAEB1	46,XY; t(12;16;18)	intermed. 2	epo
12	F	70	RAEB 2**	complex	high	no
13	M	60	RAEB2	47,XY; +8	high	no
14	F	78	RAEB 2	46,XX;del5q	high	no
15	F	72	RAEB 2	46,XX	intermed. 2	no
16	M	70	AML M4	46,XY;i(21q);del7q	N.V.	no
17	M	54	AML M2	46, XX	N.V.	no

*Diagnosis according to WHO classification. RA, refractory anemia; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RAEB 1, refractory anemia with excess of blasts type 1; RAEB-2, refractory anemia with excess of blasts, type 2; AML, acute myeloid leukemia. °Risk groups defined according to International Prognostic Score System. **Secondary to previous essential thrombocythemia.

Italy). RNA quantification was performed by UV absorbance at 260 nm.

cDNA synthesis was performed with 4 μg of total RNA in a reaction volume of 40 μl containing 1.25 μg of random primers, one mM of dATP, dGTP, dCTP and dTTP (Invitrogen, Milano, Italy), 66 units of RNAguard (Amersham Biosciences, Cologno Monzese, Milano, Italy), 8 μl of 5x first strand buffer, 10 mM DTT, and 300 units of MMLV reverse transcriptase (Invitrogen). Samples were incubated for 1 h at 37°C and the reaction was stopped by heating for 10 min at 95°C. PCR reactions were performed in a GeneAmp PCR System 9600 (Perkin Elmer, Waltham, MA USA), with 1 μl of cDNA reaction mixture in a volume of 50 μl containing 200 μM of dATP, dTTP, dGTP and dCTP, 1 μM of 5'- and 3'-primer and 1.25 units of TAQ DNA polymerase (Finnzymes, Milano, Italy). Samples were subjected to denaturation at 94°C for 30 sec, annealing (1 min at 52°C for GAPDH primer, 30 s at 64°C for sp-1 and c-myc primers, 1 min at 62°C for mad1) and extension at 72°C for 45 sec, followed by a final extension at 72°C for 3 min. Negative controls contained water instead of cDNA.

RNA analyses were performed by a semi-quantitative PCR method as previously described.[24] Briefly, the experimental strategy included the following precautions: (i) the number of PCR cycles was kept low in order to obtain an exponential amplification of PCR products; (ii) all results were standardised using the signal obtained with GAPDH (glyceraldehyde 3-phosphate dehydrogenase); (iii) all experiments were performed with at least three independent cDNA preparations; (iv) to control for DNA contamination, primers were designed to span at least one exon-intron boundary. The primer pair sequences used for PCR amplification and the numbers of PCR cycles done are indicated as follow:

hTERT-30 cycles,[25] amplifying a 175-bp fragment.

(forward primer): 5'-ACG GCG ACA TGG AGA ACA A-3'

(reverse primer): 5'-CAC TGT CAA CCG CAA GTT CAC-3'

c-myc-30 cycles,[26] amplifying a 788-bp fragment.

(forward primer): 5'-GAG ACA ACG ACG GCG GTG-3'

(reverse primer): 5'-GCT CGT TCC TCC TCT GGC-3'

mad1-30 cycles,[27] amplifying a 318-bp fragment.

(forward primer): 5'-CCA GGT GGA GCG GGA GAA AAT GC-3'

(reverse primer): 5'-CCA CTG CAG TTC CGA GAT CCT CC-3'

p53-38 cycles,[28] amplifying a 434-bp fragment

(forward primer): 5'-TCT GGG ACA GCC AAG TCT GT-3',

(reverse primer): 5'-GGA GTC TTC CAG TGT GAT GA-3',

GAPDH-25 cycles, amplifying a 119-bp fragment.

(forward primer): 5'-GTC GGA GTC AAC GGA TTT GG-3'

(reverse primer): 5'-GGG TGG AAT CAT ATT GGA ACA TG-3'

GAPDH primers were designed by using Primer Express software (Applied Biosystems, Monza, Italy). A 10 μl sample of the PCR reaction mixture was separated on a 1% agarose gel and amplification products were stained with GelStar nucleic acid gel staining (FMC BioProducts, Rockland, ME USA). Densitometric

analysis was perfomed by using a software program (Multi-Analyst, version 1.1, Bio-Rad Laboratories, Segrate, Italy).

Protein extraction and telomerase activity determination. Protein extraction. 10 x 10^6 low density BM cells were seeded and resuspended in 200 µl of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer, containing 0.5% CHAPS, 1 mM $MgCl_2$, 10% glycerol, 10 mM Tris HCl pH 7.5, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM β-mercaptoethanol (Sigma-Aldrich S.P.A., Milan, Italy). After 30 min of incubation on ice, the lysates were centrifuged at 16,000 g for 20 min at 4°C, and the supernatant was rapidly frozen and stored at -80°C.

Telomerase assay. Telomerase activity was assayed by a modification of conventional TRAP assay,[4] as described by Gelmini and coll.[29] This method is based on the use of a sensitive fluorochrome that selectively binds double-stranded DNA. Because telomerase generates double-stranded DNA from a protein extract and the amount of newly synthesized DNA is proportional to telomerase activity,[30] the measurement of DNA concentration in post-PCR samples can be considered quantitatively related to telomerase activity.[29] Each sample was assayed for telomerase activity in duplicate, starting from protein extracts of cell lines. A negative control, obtained after pre-treatment of the sample with RNase, was also assayed for each specimen. The protein concentration was measured in each extract by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Segrate, Italy). An aliquot of extract containing 3 µg of protein was used for each duplicate. RNase (Roche Diagnostic S.P.A., Monza, Milano, Italy) was used at 0.5 µg/assay for 30 min at 37°C to inactivate telomerase. Each extract was assayed in 47.2 µl of reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 4.5 mM $MgCl_2$, 1 mM each dNTP, 20 pmol of TAG-U primer,[30] and 0.5 µM T_4 gene 32 protein (Roche Diagnostic S.P.A., Monza, Italy). After 60 min incubation at 30°C for telomerase-mediated extension of TAG-U primer, the reaction mixture was heated at 90°C for 3 min and then subjected to 50–60 PCR cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 30 s, followed by 72°C for 10 min after the addition of 2.8 µl of a second reaction mixture containing 20 pmol of CTA-R primer,[30] and 0.3 µl of 5 U/µl of Taq Gold (Applera Italia, Monza, Italy). Ten microliters of each PCR product was diluted with 490 µL of 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 7.5 (Sigma-Aldrich S.P.A.), and then 500 µl of ultrasensitive fluorescent dye PicoGreen (Molecular Probes Inc., Leiden, The Netherlands; 1:1,000 diluted stock solution) was added. Fluorescence was measured in a Luminescence Spectrometer LS 55 (Perkin Elmer, Waltham, MA USA) using standard wavelengths (excitation at 480 nm, emission at 520 nm). The DNA concentration was calculated for each sample on a calibration curve generated by dilutions of a control DNA (0–100 µg/L). The final DNA concentration of each sample was obtained by subtracting the DNA amount obtained in the same specimen after RNase treatment. Telomerase activity was calculated as the mean of duplicates, expressed in terms of ng DNA/µg protein.

Statistical analysis. Unpaired Student's t-test was used to compare differences of telomerase activity, between groups. The same test was used to compare the differences in hTERT, c-myc, mad1 and p53 expression, calculated as a ratio between the specific gene and GAPDH expressions, between control and MDS groups. Comparisons of more than two groups were measured with analysis of variables (one-way ANOVA and multiple comparison). Correlations were examined by the Spearman rank correlation test. A value of $p < 0.05$ was considered statistically significant.

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