Telomerase Activity and MDS/EVI Gene Fusion in Myelodysplastic Syndrome. Correlation to the Immunohistochemical Expression of Ki-67, Bcl-2 and p53 in Bone Marrow Biopsy Samples

Rubens Jovanovik¹, Vesna Janevska¹, Lidija Cevreska², Zlate Stojanoski², Milka Zdravkovska³, Gordana Petrushevska¹

¹Institute for Pathology; ²Hematology Clinic; ³Institute of Epidemiology and Biostatistics, Faculty of Medicine, University “Ss Kiril and Metodij”, Skopje, Republic of Macedonia

Abstract

Background. Myelodysplastic syndrome (MDS) as a complex disorder comprised of 7 entities may arise as a primary disorder, or in a setting of an underlying disease, or as therapy related (secondary MDS). Some cases show MDS/EVI1 gene fusion, and some studies have pinpointed the association between the high-risk MDS and increased telomerase activity.

Aim. To determine the frequency of MDS/EVI1 gene fusion in cases of primary MDS, and to evaluate the possibility for detection of increased telomerase activity in peripheral blood samples from patients with MDS.

Material and methods. We isolated DNA from 35 bone marrow biopsies, and measured the blood telomerase activity (RTA) in 21 of the patients. We performed immunostainings for Ki-67, Bcl-2 and p53 on the biopsy samples in order to test the correlations to the RTA and MDS/EVI1 presence. MDS/EVI1 fusion was detected with touch-down-direct PCR, and RTA was measured using the “TeloTAGGG-PCR-ELISA-plus kit”.

Results. We found MDS/EVI1 fusion in 17.39% of high-risk MDS cases (overall 11.43%). RTA was highly variable in the analyzed group, with 1.8 fold increase of the mean RTA compared to the controls. It was due to the significant RTA increase in high-risk MDS cases, compared to the low-risk cases (p<0.01).

Conclusion. RTA showed correlation to the immunohistochemical expression of Ki-67, and MDS/EVI1 fusion was correlated to the Bcl-2 expression.
Basic Science

Telomerase, the enzyme that elongates telomeres, contains an RNA template complementary to TTAGGG repeats that permits de novo synthesis of telomeric DNA onto chromosomal telomeric ends. Telomerase is repressed in most normal somatic cells, but is reactivated in many of the malignant tumour cells and immortal cell lines, thus enabling greater proliferation capacity (12-14). Progressive shortening of telomeres may be the major mechanism of cellular senescence and may result in chromosomal instability and cell death as well (12,14,15). Telomerase expression and consequent stabilization of telomeres seem to be concomitant with the attainment of immortality of cancer cells (13,16-18,19, 20). At the beginning of the 90-ties several authors compared the telomerase activity in normal lymphocytes with the one in B-CLL cells, as well as with the telomerase activity in cells from other haematological malignancies including cases of myelodysplastic syndrome (1, 21-23). Most of the authors report significantly increased telomerase activity, especially in more advanced stages of the investigated diseases.

Our aim was: to evaluate the frequency of MDS/EVI1 fusion in BM samples from patients with primary MDS; and to correlate the findings with the immunohistochemical (IHH) expression of p53 (cell cycle controller), Bcl-2 (antiapoptotic protein), and Ki-67 (proliferation marker) on paraffin sections, as well as with the telomerase activity in patients’ peripheral blood samples taken at the time when the diagnosis was histologically confirmed.

Material and methods

Bone marrow biopsy samples

We analyzed 35 formalin-fixed paraffin-embedded (FFPE) BM samples (trephine biopsies) from patients diagnosed as primary MDS according to the WHO and FAB diagnostic criteria (1, 2, 24). All the samples were consecutive cases submitted at the Institute for Pathology during years 2003 and 2004 from the Clinic for Haematology. The distribution of the samples according to the types of MDS was as follows: RA (n=12), RAEB (n= 11), RAEBt (n=10), and MDS/CMMLt (n= 2).

Immunostaining

All BM samples were immediately immersed in a mixture of 10% neutral formalin and decalcination agent after the biopsy, and afterwards transported to the Institute of Pathology. Samples were processed in a tissue processor and embedded in paraffin blocks.
Five to seven μm thick sections were stained with H.E., Giemsa, PAS, and Reticulin. Immunostainings (LSAB+; DAKO) for p53 [clone DO-7, DAKO], Bcl-2 [clone 124, DAKO], and Ki-67 [clone MIB-1, DAKO] were also performed. Antibody dilutions were as follows: p53 (1:25), Bcl-2 (1:50), and Ki-67 (1:25). IHH stainings were evaluated in a semi quantitative manner on 4 sets of 10 high power magnification fields (4x10 hpmf). The evaluation thresholds for all of the markers were as follows: (1+) 0-10% of cells expressing nuclear signal for Ki-67 and p53, and cytoplasmic signal for Bcl-2; (2+) 11-50% of cells expressing nuclear signal for Ki-67 and p53, and cytoplasmic signal for Bcl-2; and (3+) >50% of cells expressing nuclear signal for Ki-67 and p53, and cytoplasmic signal for Bcl-2 (Fig. 1). Samples expressing positive signal in only 0-10% (1+) were considered negative, or not having increased expression.

**Telomerase activity**

Peripheral blood samples were obtained for telomerase activity evaluation from 22 of the patients. We were unable to test the telomerase activity in BM samples since all of them were previously fixed according to the standard procedure. As a control for the telomerase activity, we used the data from our earlier study, which included a control group of 47 healthy subjects, with non-significant age difference with the present examination group.

The telomerase activity was analyzed with commercially available kit “Telo TAGGG PCR-ELISA plus” (Roche). Analyses were conducted according to the manufacturer’s recommendation without any modifications. The results were expressed as relative telomerase activity (RTA).

**PCR detection of MDS1/EVI1 fusion**

We isolated DNA for MDS1/EVI1 gene fusion detection from paraffin sections. We applied hot start - touch down – direct PCR, with the following primers: P1: CCCATCTACATCCCTGATG (start: pos. 181/ex1 MDS1), and P2: CTGATCATTTATCTGGCATG (end: pos. 135/ex2 EVI1). Primers were designed using GeneRunner software V3.05, and referent sequences for MDS1 and EVI1 (ENSG00000206115; ENSG00000085276 – Database ver. 41.36c), with subsequent testing of the primers by means of Virtual PCR (25), including testing of the sequence, length, and specificity (Ensemble/BLAST; PRIMEX). The expected product length is approximately 300 bp; (~283 - 318 bp) depending on the exact site of gene fusion.

The PCR program we used was as follows: Hot start 95°C, 6 min; touchdown: 56.3–61.3°C/10 cycles; start cycles: eukaryotic/35 times; denaturation 95°C, 30 s; annealing 61.3°C, 30 s; elongation 72.0°C, 60 s; store 4°C. The PCR products were analyzed after electrophoresis on 1.5% agarose gels containing ethidium bromide and subsequent visualization on UV light (Fig. 2).
Statistical analysis

For description of the obtained results, methods from the descriptive statistics were used and the null hypothesis was tested using parametric and non-parametric tests by means of commercial software “StatSoft, Inc. STATISTICA (data analysis software system) version 6”. The normality of distribution was tested using the Kolmogorov-Smirnov D test, Lilliefors test and Shapiro-Wilk’s W test. We used t-test for independent samples and Mann-Whitney U test for testing the mean. The significance of differences between patients’ subgroups was evaluated by means of nonparametric tests for comparison of multiple independent samples (Kruskal-Wallis ANOVA by ranks and Median chi-square test). Correlations between parametric variables were tested with Pearson Product Moment Correlation (r), and for the non-parametric variables the Spearman’s Rank Order Correlation (R) was used.

Results

The examined group (n=35) showed the following features: 20 (57.1%) were males and 15 (42.9%) were females, with mean age 58.3 years (min. 38; max. 82; SD=11.6). The distribution of the patients according to the type of MDS was as follows: RA 34.28% (n=12), RAEB 31.42% (n=11), RAEBt 28.57% (n=10), and MDS/CMMLt 5.71% (n=2). There were no patients with MDS-RARS and MDS-CMML in this series (Table 1).

Table 1: Descriptive statistics of the analyzed group.

<table>
<thead>
<tr>
<th></th>
<th>Males (count)</th>
<th>Females (count)</th>
<th>Age (years) (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All MDS cases</td>
<td>20</td>
<td>15</td>
<td>58.3±11.6 (min. 38; max. 82; median 57.5)</td>
</tr>
<tr>
<td>RA</td>
<td>7</td>
<td>5</td>
<td>61.3±12.4 (min. 41; max. 82; median 62)</td>
</tr>
<tr>
<td>RAEB</td>
<td>6</td>
<td>5</td>
<td>58.8±11.3 (min. 41; max. 77; median 58)</td>
</tr>
<tr>
<td>RAEBt</td>
<td>5</td>
<td>5</td>
<td>55.4±9.2 (min. 43; max. 71; median 52)</td>
</tr>
<tr>
<td>CMMLt</td>
<td>2</td>
<td>0</td>
<td>56±25.4 (min. 36; max. 74; median 56)</td>
</tr>
</tbody>
</table>

The type of MDS showed no correlation to the patients’ age and gender. The age and gender of the patients showed no influence on the RTA and the expression of IHH markers as well.

The relative telomerase activity (RTA) in the analyzed group was highly variable. There was significant 1.8 fold overall increase in mean RTA (16.6; min. 6.9; max. 34.1; SD=7.2) compared to the values of the healthy controls (9.2; min. 2.1; max. 24.2; SD=5.3) (t = -4.67; DF=16; p<0.01). We found no significant inter-group differences in mean RTA between different types of MDS (Fig. 3).

As for the difference in mean RTA between the control group and MDS subgroups, we detected significant mean RTA increase in RAEB samples (mean=17.3; SD=4.9) and RAEBt (mean=18.5; SD=6.8), compared to the control mean RTA (M-W U test: U=24; Z=3.2, p<0.01; and U=9; Z=2.8; p<0.01 respectively). The mean RTA in RA patients (10.8; SD=3.9) showed no significant increase compared to the control mean RTA (p> 0.05). We were unable to statistically evaluate the RTA from MDS-CMMLt samples because of their low number in the examined group, although those samples presented highest RTA and influenced the
overall increase of the mean RTA in the group of high-risk MDS cases (RAEB, RAEBt, CMMLt).

When we divided the patients into subgroups of low risk MDS (RA) and high risk MDS (RAEB, RAEBt, CMMLt) (19), significant differences appeared between the mean RTA in the first (mean=10.8; SD=3.9) and the second subgroup (mean=19.1; SD=6.95) (p<0.01) (Fig. 4).

![Fig. 4: RTA in low- and high risk MDS patients. Mann-Whitney U test: U= 11; Z=-3.2; p<0.01.](image)

There was no significant difference between the RTA in MDS/EVI1 fusion positive MDS cases compared to the RTA in MDS/EVI1 negative ones.

RTA showed positive correlation to the IHH expression of Ki-67 (Spearman R = 0.44; p<0.05), but not to the other IHH markers. MDS cases over expressing Ki-67 had significantly higher RTA (mean=19; SD=8.3; min. 9.9; max. 34.1) compared to those with 1+ Ki-67 expression (mean=12.5; SD=4.4; min. 6.9; max. 21.5) (p<0.05) (Fig. 5).

Table 2 shows the frequencies of the rest of the examined parameters.

<table>
<thead>
<tr>
<th>Intensity</th>
<th>1+</th>
<th>2+</th>
<th>1+</th>
<th>2+</th>
<th>1+</th>
<th>2+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11/12 (91.7%)</td>
<td>9/12 (75%)</td>
<td>12/12 (100%)</td>
<td>10/12 (83.3%)</td>
<td>12/12 (100%)</td>
<td>12/12 (100%)</td>
</tr>
<tr>
<td>RAEB</td>
<td>9/11 (81.8%)</td>
<td>2/11 (18.2%)</td>
<td>3/11 (27.3%)</td>
<td>8/11 (72.7%)</td>
<td>10/11 (90.9%)</td>
<td>11/11 (100%)</td>
</tr>
<tr>
<td>RAEBt</td>
<td>8/10 (80%)</td>
<td>2/10 (20%)</td>
<td>5/10 (50%)</td>
<td>6/10 (60%)</td>
<td>9/10 (90%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>CMMLt</td>
<td>1/2 (50%)</td>
<td>1/2 (50%)</td>
<td>2/2 (100%)</td>
<td>2/2 (100%)</td>
<td>2/2 (100%)</td>
<td>2/2 (100%)</td>
</tr>
<tr>
<td>Total MDS</td>
<td>20/35 (57.1%)</td>
<td>11/35 (31.4%)</td>
<td>19/35 (54.3%)</td>
<td>22/35 (62.9%)</td>
<td>23/35 (65.7%)</td>
<td>24/35 (68.6%)</td>
</tr>
</tbody>
</table>

*We found no 3+ IHH positivity for none of the tested markers.

We detected MDS/EVI1 gene fusion in 4 of the high risk MDS with a frequency of 1/11 of RAEB cases, 2/10 of RAEBt cases, and one of the MDS-CMMLt cases, or 17.4% of high risk MDS cases and overall 11.4% of cases (Tab. 2). There was a positive correlation between the Bcl-2 over expression and the presence of MDS/EVI1 fusion (R = 0.62; p<0.01), with a significant expression difference between MDS/EVI1 negative cases compared to MDS/EVI1 positive cases (Yates corrected Chi-square = 8.6; p< 0.01; Fisher exact p<0.01). We also found positive correlation between Ki-67 and the expression of the antiapoptotic protein Bcl-2 (Spearman R = 0.37; p<0.05), as well as between Ki-67 and p53 (Spearman R = 0.66; p<0.01), but there was no correlation between Bcl-2 and p53.

Discussion

The descriptive statistical analysis of the examined group showed age and gender distribution similar to the ones described in literature (1, 2, 26, 28). RTA in our series is comparable with that published by other authors (20, 26). The RTA increase in MDS compared to the controls, emerges from the RTA increase in the subgroup of high risk MDS (in our case: RAEB, RAEBt, CMMLt), while the mean RTA in the low risk MDS group (in our case only RA) showed no significant difference compared to the mean RTA in the control group. It is very important to emphasize that the obtained RTA values were from peripheral blood samples, unlike the published data from Fu et al., 2002 (26) who measured telomerase activity in bone marrow samples. If this data could be reproduced on a larger series, it would mean potential introduction of an additional parameter for disease monitoring since, unlike BM biopsy, peripheral blood samples may be more easily and repeatedly obtained. The study of Ohyashiki et al., 1999 (27), who measured RTA both
in BM and peripheral blood mononuclears, has confirmed that there was no significant difference. Another rather logical result was the correlation between the RTA and the IHH expression of Ki-67, leading to a conclusion that the proliferative activity in BM affects the telomerase activity in blood mononuclears. However, the question remains whether the RTA increase is solely a result of the discharge of immature precursors from BM, or there is some residual telomerase activity (and/or telomerase re-activation) in mature blood mononuclears, having in mind that theoretically, because of the ineffective BM haematopoiesis and increased proliferation, even mature precursors could have critically shortened telomeres.

In our series we detected the MDS/EVI1 gene fusion only in high risk MDS cases (1/11 of RAEB cases, 2/10 of RAEBt cases, and one of the MDS-CMMLt cases). With overall frequency of 4/35 (11.4%), this finding was far more frequent in our series than in the published study of Hirai, 2003 (30), who reported only 2% of cases having structural aberrations involving loci 3q21/26. This difference could be partly due to the difference between the examination groups. We only investigated patients with primary MDS, and it is well known that those with secondary MDS have more complex and variable genetic aberrations (2, 30), thus virtually lowering the overall frequency of this particular gene fusion. The detection of MDS/EVI1 gene fusion might be useful complementary analysis to the standard clinical, laboratory and histological parameters for confirmation of at least some of the high-risk primary MDS patients. Opposite to this point of view, stands the study of Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003 (3), which partially minimizes the importance of MDS/EVI1 gene fusion detection in patients with MDS. In that study, the importance of MDS/EVI1 gene-fusion product quantification is stressed-out, as well as the necessity for discrimination between the gene-fusion product variants: EVI1 gene product, MDS-EVI1 gene product, and MDS gene product, giving the greatest significance to the over-expression of EVI1 as a negative prognostic marker. However, the above stated study (3) was performed on a series of 319 cases of de novo acute myeloblastic leukaemia (AML), and did not include classic MDS and cases of MDS in transformation to acute leukaemia. Another study, conducted by Lugthart et al. 2008 (5), also highlights the over-expression of EVI1 (most frequently splice variant EVI1-1D) as a negative indicator in AML cases, especially if it is not co-expressed with MDS1/EVI1 (ME). Lugthart et al., 2008 (5) have shown significantly inferior outcome for EVI1+/ME- AML patients in comparison with EVI1+/ME+ patients, in a cohort of 41 EVI1+ AML patients. They have also shown that such dissociated EVI1 expression (EVI1+/ME-) is most frequently associated with cytogenetically and/or FISH detectable 3q26 abnormalities and -7/-7q- deletion, while EVI1+/ME+ AML cases frequently carry 11q23 abnormalities, suggesting possible influence of other fusion genes on EVI1 and ME transcription. Unlike Lugthart et al., 2008 who did not find any structural 3q26 aberrations in EVI1+/ME+ AML patients (n=41), we detected MDS/EVI1 fusion in 4 out of 35 primary MDS cases (all of them high risk MDS). This difference could be a result of the pathogenetic differences between primary MDS and AML, and may be this gene fusion, found at least in a small proportion of high risk MDS patients, that prevents dissociated EVI1 and MDS/EVI1 expression (EVI1+/ME-), characteristic of the most unfavourable subgroup of AML. This implies enlargement of our cohort and assessment of MDS1/EVI1 and EVI1 expression in order to evaluate the possible influence of MDS1/EVI1 fusion on the evolution of MDS1/EVI1+ and MDS1/EVI1- high risk primary MDS cases, and possible differences between this two subgroups.

One of the major disadvantages of our study, compared to the above cited studies (3, 5), besides the relatively small cohort, is the lack of (quantitative) expression analysis of MDS1/EVI1 and EVI1 (i.e. EVI1-1A, -1B, -1C, -1D, and 3L), as proven prognostic markers in AML, although the main target of our study was primary MDS.

The IHH expressions of Ki-67 and p53 were not correlated to neither MDS/EVI1 fusion, or to the low- and high-risk MDS subgroups. However, we found positive correlation between the Bcl-2 over expression and the presence of MDS/EVI1 fusion (R = 0.62; p<0.01), with a significant IHH expression difference between MDS/EVI1 positive cases compared to MDS/EVI1 negative cases (Yates corrected Chi-square = 8.6; p< 0.01). This leads to a conclusion that the MDS/ EVI1 fusion positive MDS variants express higher anti-apoptotic activity. Unlike the study of Davis and Greenberg, 1998 (31), we found no correlation between the Bcl-2 over expression and the advanced MDS (high-risk) subgroup. However, in the cited study the authors do not provide data about the MDS/EVI1 status of the examined subjects. We also found positive correlations between Ki-67 and the expression of the antiapoptotic protein Bcl-2 (R = 0.37; p<0.05), as well as between Ki-67 and p53 (R = 0.66; p<0.01), but there was no correlation between Bcl-2 and p53. We consider performing further stainings on larger cohort, since the evident cross-correlations between Ki-67, Bcl-2, and p53 could turnout to be directly correlated.
to the high risk MDS as well. Another motivation for further analysis are the published data about mutations, inactivation or deletion of both p53 alleles in 5-10% of MDS cases, especially in high risk ones (30).

We can conclude that:

1. The mean RTA in peripheral blood samples from high risk MDS patients is significantly higher compared to low risk MDS patients and healthy controls (p<0.01).

2. There is no significant RTA difference in blood samples from low risk MDS patients compared to healthy controls.

3. Peripheral blood RTA is in positive correlation with the IHH expression of Ki-67 in bone marrow (Spearman R = 0.44; p<0.05), but not with Bcl-2 and p53.

4. MDS/EVI1 gene fusion was detected in FFPE BM biopsies in 17.4% of high risk MDS cases, with overall frequency of 11.4% of all examined cases.

5. There is a positive correlation between the IHH over-expression of the anti-apoptotic protein Bcl-2 and MDS/EVI1 fusion (R = 0.62; p<0.01), with a significant expression difference between MDS/EVI1 negative and MDS/EVI1 positive MDS cases (Yates corrected Chi-square = 8.6; p< 0.01).

Acknowledgments

This study has been completed as a part of the Tempus-Phare postgraduate program “Restructuring the postgraduate studies in molecular biology and genetic engineering at the University of Skopje (S-JEP-1220-97), Interdisciplinary studies in molecular biology and genetic engineering – Molecular medicine course,” under auspices of the University St. Kiril and Metodij in Skopje, Republic of Macedonia.

References


PMID:12393383

PMID:8171026

PMID:18272813


PMID:9447818


PMID:9447818

PMID:16014322

PMID:10963374


