p53 PROTEIN AS A SURVIVAL BIOMARKER IN MYELODYSPLASTIC SYNDROMES: IMMUNOHISTOCHEMICAL MORPHOMETRIC STUDY

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Abstract

Patients with myelodysplastic syndromes (MDS) show highly variable clinical course. The average overall survival is 15-30 months, and the risk of acute myeloid leukaemia (AML) transformation is 25-30% after five years. The molecular mechanisms of leukemic progression and transformation are still incompletely understood. TP53 mutation frequency in MDS is 5-10%, these being associated to all clinical forms of the disease, determining a shorter overall survival. The objective of our study was to evaluate the relationship of p53 protein expression with survival and AML transformation in MDS patients. We quantified the p53 levels by the means of immunohistochemical staining and a digital morphometric approach in MDS bone marrow biopsy specimens. In our cohort, a higher p53 protein expression was observed in the high leukemic transformation risk group. p53 expression, together with the bone marrow blast count, proved to be a significant risk predictor when analysing survival.

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Keywords: biomarker, myelodysplastic syndrome (MDS), p53 protein, digital morphometry

Introduction

Myelodysplastic syndromes (MDS) are clonal haematopoietic stem cell disorders, characterized by ineffective haematopoiesis, due to uni- or multi-lineage dysplasia and various combinations of anaemia, leukopenia and thrombocytopenia. Patients with myelodysplastic syndrome show highly variable clinical course, ranging from silent disease to very aggressive forms with quick transformation in acute myeloid leukaemia (AML) [9]. The average overall survival is 15-30 months, and the risk of AML transformation is 25-30% after five years [4]. Excess blasts are the strongest predictor for a poor prognosis, but according to the WHO 2008 classification, the risk of AML transformation in myelodysplastic syndromes is determined also by histological subtype [13] and the laboratory, phenotypical, cytogenetic and molecular factors included in the International Prognostic Scoring System (IPSS) criteria, published in 1997, revised in 2012 [6, 7]. This standard for assessing prognosis of primary untreated MDS confirmed that the number of blasts and cytogenetic subgroups are significant prognostic factors for AML transformation, contrary to the patient age, performance status, serum ferritin and lactate dehydrogenase activity- significant additive features for survival, but not for AML transformation [6]. One important limitation of the IPSS criteria is that more than 50% of patients with myelodysplastic syndrome have a normal karyotype [3]. In these cases, application of other biomarkers is desirable to predict the disease course outcome. The molecular mechanisms of leukemic progression and transformation are still incompletely understood, but a two-hit model of MDS-AML interface (MDS transformation in AML) is currently supported. According to this model, Class I targeted genes (Flt3, RAS, KIT, p53) are involved in signal
transduction, while mutations in Class II genes (TET2, AML/RUNX, RSP14) affect critical transcription factors. Nolte et al. demonstrated, that both Class I and Class II mutations may result to primary AML [10]. TP53 mutation frequency in MDS is 5-10%, and was found in both low-risk, intermediate-, high-risk and therapy-related MDS [3, 4]. These mutations are strongly associated with thrombocytopenia, elevated blast proportion, and a complex karyotype, determining a shorter overall survival [1]. Strong immunoeexpression of p53 protein in myeloid cells in the bone marrow of MDS patients has been shown to precede MDS-AML transformation [8]. A substantial heterogeneity of disease course is characteristic for currently defined patient risk-groups that emphasizes the requirement for improved prognostic algorithms. The objective of this study was to evaluate the role of p53 protein expression in estimation of survival and AML transformation by the means of immunohistochemical staining and morphometric quantification in MDS bone marrow biopsy (BOM) specimens. The rate of p53 positive nucleated cells of myeloid lineage was quantified by digital morphometry measuring the extent of p53 positive area.

**Materials and Methods**

**Patients and Bone marrow histology**
In this retrospective study all BM trephine biopsy specimens with morphological characteristics of MDS (diagnosed in the Emergency Clinical Hospital, Târgu-Mures, Romania, between 2010 and 2015) were included. Secondary MDS was an excluding criterion. Patients’ data (age, sex, clinical evolution) were collected from the clinical observation sheets. Following the review of these cases (based on the bone marrow findings – unilineage or multi-lineage dysplasia, blast and ringed sideroblast count, presence or absence of the “abnormal localization of the immature precursors” (ALIP), the final database was restricted to 47 BM trephine biopsies. According to the WHO criteria these were classified as refractory anaemia (RA), refractory cytopenia with multi-lineage dysplasia (RCMD), refractory anaemia with blast excess (RAEB-1, RAEB-2) [13]. All diagnoses were confirmed independently by two pathologists. Because cytogenetic data were unavailable for the majority of patients, we did not apply the IPSS classification for risk assessment in the different MDS subtypes. Instead, we split the MDS cases in two risk-groups: a low-risk group including cases with blast count < 5% (RA, RCMD) and high-risk group, including cases with blast count > 5% (RAEB-1 and RAEB-2). The control group was represented by 10 patients with non-Hodgkin lymphoma (who were investigated as part of the staging procedure) but no evidence of bone marrow involvement.

The following parameters were studied: cellularity, blast count, presence of ALIP, p53 positive cell rate (p53c), p53 positive nuclear surface (p53s), survival time, evolution and mortality. The study was approved by the Ethics Committee of the Emergency Clinical Hospital, Târgu-Mures.

**Immunohistochemical examination: study of p53 protein expression in blast/blast equivalent cells**
The p53 protein expression of the myeloid cells was assessed by immunohistochemistry using a monoclonal p53 antibody (clone DO-7, Dako, 1:50 dilution) and the heat-induced antigen retrieval method (in the microwave oven). The reaction product was visualised, applying the DAKO En Vision TM Flex, High pH immunodetection system (K8010) in combination with 3,3'-diaminobenzidine chromogen (DAB) followed by haematoxylin counterstain. Staining was optimised using positive and negative controls. As positive control we used grade 3 colon carcinoma histological samples. As negative control, the primary antibody was replaced by normal serum.

**Morphometric analysis**
The rate of p53 positive nucleated cells of myeloid lineage was quantified by digital morphometry measuring the extent of p53 positive area. For each case, five microscopic fields were chosen for quantification (“hot spot” method), excluding the cortical and trabecular bone, periosteal connective tissue, haemorrhagic foci and areas occupied by fat tissue. These microscopic fields were captured, using the Mirax Digital Slide Scanner image acquisition system for image processing (Panoramic Viewer 1.15.4). We photographed the selected representative fields with 20x objective, saved the digital images in JPEG format and imported them for analysis into the Image J software [12] (Figure 1). In the first phase we selected the p53 positive nuclei using colorimetric segmentation, which results in a colour image. After the colorimetric segmentation, the selected fields (ready for fully automated analysis) contain only the desired tissue structures (in our case the p53 positive areas). The immunohistochemical staining greatly enhances the potential of colorimetric segmentation, since the DAB stain most of the time stands out clearly from the rest of the tissue (performed manually). The second phase consists of the measurement of the p53 positive areas, preceded by the conversion of the image from 24-bitRGB format to 8-bit black and white format (Figure 4: 8-Bit B&W image as the final step of the automated analysis). This latter format is ready for fully automated analysis, as the software automatically records the p53 positive areas in a spread sheet next to the file name (Figure 2).
Figure 1.
Image processing and analysis with Image J software

Figure 2.
a. BM trephine biopsy (H&E staining, 1X): hyper-cellular MDS, RAEB-2. b. Bone marrow section with abnormal localization of immature precursor(ALIP) and multi-lineage dysplasia(H&E stain, 40 x), c-f: Selection of 4 images captured with 20 x objective, (p53 in marrow, DAB stain) preliminary to the determination of p53c and p53s by digital morphometry (Image J) using the “hot spot” method
The final result is calculated individually for each case based on the arithmetic mean. Two p53 measures were determined: the p53c (cell count) meaning the proportion of the p53 positive nuclei in the analysed area, and p53s, which summarized the p53 positive relative surface.

We used the p53 expression levels to adjust the leukemic transformation risk classification correcting the risk group of low blast count, but upper quartile p53s expression cases into high-risk, and the group of high blast count and lower quartile p53s expression into low-risk category.

**Statistical analysis**

Data were analysed with the OpenStat and STATISTICA 5.0 softwares. Continuous variables, as demographic data and microscopic findings were analysed for distribution with the Kolmogorov-Smirnov and Lilliefors tests. With the exception of age these showed abnormal distribution and consequently were analysed by nonparametric tests: the Mann-Whitney U test, and the Spearman correlation. The effect size (r) was calculated and represented for each Mann-Whitney comparison. Data with abnormal distribution were represented as median and quartile range. Discrete variables were analysed by the Fisher exact test. Kaplan-Meier survival analysis was performed for the low versus high transformation risk group comparison.

**Results and Discussion**

The 47 myelodysplastic syndrome cases were classified as follows: refractory anaemia (RA): 11 (23.4%), refractory anaemia with blast excess -1 (RAEB-1): 12 (25.5%), refractory anaemia with blast excess -2:17 (36.2%), refractory cytopenia with multi-lineage dysplasia (RCMD): 7 (14.9%).

The main demographic, clinical and laboratory parameters of the group are shown in Table I.

<table>
<thead>
<tr>
<th>Myelodysplastic syndrome patient group characteristics (n = 47)</th>
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<tbody>
<tr>
<td>Age (years, mean ± SE)</td>
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<tr>
<td>Gender (male/female, no.)</td>
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<tr>
<td>Low/High transformation risk (no.)</td>
</tr>
<tr>
<td>Cellularity (hypo/normal/hyper-cellular, no.)</td>
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<tr>
<td>Blast count category (C1/C2/C3)</td>
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<tr>
<td>ALIP (no/yes, no.)</td>
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<tr>
<td>p53 cell count (% m.f.)*</td>
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<tr>
<td>p53 expression (% surface)*</td>
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<tr>
<td>Survival (months)*</td>
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Values expressed as no. = number, SE = standard error of the mean, *median (quartile range)

Mean age of the group was 60.38 ± 1.77 years and by gender more males (26, 55.3%), than females (21, 44.7%) were enrolled. By the transformation risk, 18 patients could be classified to the low-risk (LR) and 29 to the high-risk (HR) subgroups. The majority of patients presented a hyper-cellular or normal cellular bone marrow at biopsy, only 5 cases being hypo-cellular, while 29 of them showed abnormal localization of immature precursors (ALIP). Blast count (BC) distribution was almost equal in the three lower subgroups (C1-blasts < 5%, C2-blasts 5-10%, C3-blasts > 10%).

At the overall group, there was a significant positive correlation between the two p53 expression measures (p53c and p53s): r = 0.64, p < 0.001. p53c showed higher relative values, than p53s.

**p53 expression in BM biopsies**

p53c and p53s both were significantly higher in MDS patients than in controls (medians 7.70 vs. 1.62, p < 0.001, r = 0.48 and 2.70 vs. 0.54, p < 0.001, r = 0.50 respectively). Comparing the LR and HR subgroups, significantly higher p53c – 4.49 (2.31-7.92) vs. 9.16 (4.52-20.83), p = 0.017, r = -0.34 and p53s values – 0.65 (0.16-1.35) vs. 3.28 (1.15-8.30), p < 0.001, r = -0.47 were detected in HR individuals. The p53s, but not the p53c was also significantly higher in patients with acute leukemic transformation (p = 0.021, z = -0.39) (Figure 3).
Outcome comparison of TR risk subgroups

The registered survival range was 1-42 months (minimum-maximum) and during the 42 months study follow-up, 14 patients have died. The clinical evolution was stationary in 25 (53.2%) cases, 9 cases (19.1%) presented disease aggravation and other 9 (19.1%) leukemic transformation. Due to various reasons, after 1-30 months 5 cases were lost from the register, these cases were censored in the Kaplan-Meier analysis. Comparing the survival periods of patients from the LR group with the HR group, who completed the follow-up we found that the two groups despite of longer periods in the LR group: 16 (3-36) months vs. 7.5 (4-19) months, did not differ statistically \( p = 0.22, \; r = 0.19 \).

Comparing the mortality rates, 3 cases from 14 (21.4%) in the LR group and 11 from 29 cases (37.9%) in the HR group were lost. The difference was not statistically significant \( (p = 0.32) \). According to the adjusted transformation risk criterion, 22 cases felt in the LR\textsubscript{adj} group, while 25 patients could be classified to HR\textsubscript{adj}. This reclassification resulted a major shift in the survival comparison: 22 (8-36) months in the LTR\textsubscript{adj} vs. 6 (3.5-12.5) months in the HTR\textsubscript{adj}, \( p = 0.008, \; r = 0.40 \) (Figure 4a-b).
Kaplan-Meier curve representation of patients’ survival by transformation risk and adjusted transformation risk groups. HR group represented with blue line (“experimental”), LR group represented with red line (“control”), time represented in months. Number of censored cases shown for each group.

We performed the Kaplan-Meier survival analysis with both transformation risk classifications. With LR/HR, the two curves were significantly different: risk 0.511 (0.279-0.939), $\chi^2 = 4.36$, Log-rank test $p = 0.023$. Applying the adjusted risk classification, LTR\textsubscript{adj}/HTR\textsubscript{adj}, a more significant difference was observed: risk 0.478 (0.257-0.889), $\chi^2 = 9$, with a Log-rank test $p = 0.002$.

MDS is a clinically heterogeneous disorder. Many studies have demonstrated that several pathway-associated morphological, clinical and molecular parameters affect the overall survival [16]. MDS is predominantly a disease of the elderly population. The median age at the diagnosis is approximately 70 years with a male predominance [4]. In our study the mean age of the group was 60.38 ± 1.77, due to 7 cases (14.9%) younger than 50 years, slightly above the general representation of this age category (10%) [5]. 20-30% percent of cases transformed into AML. The transformation is associated with several prognostic factors: the MDS histological category, the IPSS, blast count and aberrant phenotypes in the BM blast population, cellularity, complex karyotypes and the Class I-II mutations [11]. Cases with a favourable disease course, possessing low IPSS suffer a leukemic transformation after an average of 9.4 years, and those with intermediary risk in a range of 1.1-3.3 years; while high risk cases present rapid aggravation and a mean survival of only 0.2 years. Above 60 years the survival rate decreases to an average of 4.8 years [7]. Due to this important
survival variability, in order to result in more exact, individualized prognosis, additional molecular markers should be developed and validated. Along elevation of the blast count, the appearance of p53 protein acts as a danger signal. Point mutations of p53 are characteristic for 5-20% of MDS de novo, 25-70% of t-MDS (therapy related MDS), and are transformation-related risk factors [15].

Bejar et al. using a combination of genomic approaches to identify mutations in BM aspiration samples from patients with MDS, found that the mutated TP53 gene is associated with specific clinical features of the disease: poor overall survival and severe thrombocytopenia. Patients with TP53 mutation compared with those without mutation have elevated BM blast percentages [1]. TP 53 is a tumour suppressor gene located on the short arm of chromosome 17. Its product plays two important roles in DNA-damaged cells, namely, proliferation arrest and apoptosis induction. Wild-type p53 protein has a short lifetime and cannot be detected in contrast to the mutant variant that can be visualized and quantified by immunohistochemistry on the BM tissue sections [14]. Detection of tumour markers has an important role in screening, diagnosis and outcome prediction of malignancies [17].

In our cohort, a higher p53 protein expression was observed in the high leukemic transformation risk group. This risk classification was based on the BM blast count, and morphometry revealed p53 staining of blasts and blast equivalent cells. No significant differences between-groups were detected regarding the net survival times. However, the Kaplan-Meier analysis of the groups revealed significantly lower survival times for the high risk groups. When we included the risk group alignment based on p53 expression this proved to be useful in a more exact estimation of survival period, since the adjusted high vs. low leukemic transformation risk groups showed a stronger statistic difference. These results raise the possibility of p53 participation in the pathways involved in the progression of MDS. P53 protein expression in our assays was determined by quantitative digital morphometry. The calculation of p53 positive surface approach proved to be superior in survival estimation than counting of positively stained cells TP53 genotyping may provide more complex associations with the disease outcomes.

Conclusions

According to our results, a higher p53 protein expression was associated with a shortened survival in MDS patients. A simple risk classification based solely on the blast count could separate the disease transformation risk groups. Leukemic transformation certainly confers to MDS a poorer outcome. Adjustment of the risk classification with the p53 expression leads to the change of risk class in some cases, since low blast counts sometimes may be associated with a higher p53 level, and inversely, high blast numbers may appear with a low p53 expression. p53 possibly plays a pathogenic role in MDS, and its determination could bring benefits not only in fine-tuning the prognosis, but also in the definition of the future therapeutic choice.

References


