

## The assessment of proliferating cell nuclear antigen (PCNA) immunostaining in myelodysplastic syndromes and its prognostic significance

MG. Alexandrakis,<sup>1</sup> FH. Passam,<sup>3</sup> K. Dambaki,<sup>2</sup> F. Nalbanti,<sup>4</sup> ES. Stathopoulos,<sup>2</sup> DS. Kyriakou<sup>5</sup>

Departments of <sup>1</sup>Hematology and <sup>2</sup>Pathology, University Hospital of Heraklion, Medical School of Crete, Crete; <sup>3</sup>Hematology Unit, 3rd Dept of Internal Medicine, Sotiria Hospital, Medical School of Athens, Athens; <sup>4</sup>Dept of Hematology, Hippokrateion General Hospital, Thessaloniki; <sup>5</sup>Dept of Haematology, University Hospital of Larisa, Medical School of Thessalia, Greece

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Several prognostic factors for patients with myelodysplastic syndromes (MDS) have been identified in previous years. In order to determine prognostic factors characterizing haematopoietic cell kinetics, bone marrow proliferative activity and serum TNF- $\alpha$  levels were measured in 51 cases of MDS. Cell proliferation was evaluated by employing a monoclonal antibody directed against the proliferating cell nuclear antigen (PCNA). The PCNA proliferating index (PCNA PI) and serum TNF- $\alpha$  levels showed significant differences between patients with MDS and normal controls ( $p < 0.0001$ ). PCNA PI and serum TNF- $\alpha$  were significantly higher in the high risk for leukemic transformation FAB subgroups (RAEB, RAEB-t and CMML) in comparison to the low risk group (RA and RARS) ( $p < 0.001$ ). PCNA PI and TNF- $\alpha$  also increased with increasing IPSS score ( $p < 0.05$ ). A positive correlation was noted between TNF- $\alpha$  concentrations and PCNA PI ( $r: 0.36$ ,  $p < 0.008$ ). Univariate analysis using the log-rank test showed that a higher PCNA PI was associated with a significantly shorter survival ( $p < 0.001$ ). We conclude that elevated PCNA PI and TNF- $\alpha$  serum levels are increased in high risk myelodysplastic disease and that a high PCNA PI is predictive of a shorter survival in this group of patients.

Key words: Myelodysplastic syndromes (MDS), proliferation, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), PCNA, survival

Correspondence: Michael G Alexandrakis, MD, Assist Professor of Medicine, Department of Hematology, University Hospital of Heraklion, P.O. BOX 1352 Heraklion, Crete, Greece.  
Tel: +30 2810 392426  
Fax: +30 2810 244883  
E-mail: freda@med.uoc.gr

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The myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal haematologic malignancies characterized by abnormal differentiation and maturation of myeloid cells, bone marrow failure with frequent evolution to secondary leukemia (Bennett 1995, Wimazal 2001). The precise cause of this leukemic transformation is still unknown, but it is generally held that a number of events lead to progressive changes in the abnormal clones (Stephenson 1993, Yoshida 1995). One property of the immature blastic cells that distinguishes them from their more mature counterparts is their higher *proliferative potential* or else their ability to undergo many cycles (Kitagawa 1993).

Several proteins have been identified that are specific for cycling versus quiescent cells. Monoclonal antibodies against such proteins have been produced in order to provide an accurate estimation of the growth fraction in malignant tumors (Kitagawa 1993, Lin 2002, Kyriakou 1996, Miyachi 1978). Among these, the proliferating cell nuclear antigen (PCNA) is an evolutionarily highly conserved 36-kD nuclear acidic protein, the levels of which correlate directly to the rate of cellular proliferation and DNA synthesis (Miyachi 1978, Takasaki 1981). In contrast to resting cells that synthesize a very small amount of PCNA, transformed cells synthesize PCNA in amounts that are related to the degree of transformation (Celis 1987, Bravo 1986). Therefore the examination of PCNA expression of the bone marrow could be expected to be an informative adjunct to histological diagnosis and an important indicator of the biology of the haematological disorder.

MDS usually present with variable cytopenias, tri-lineage dysplasia in generally hypercellular marrows. The contrast of peripheral cytopenias versus hypercellular marrows has been attributed to the

increased intramedullary apoptotic mechanism in MDS. TNF- $\alpha$  is a highly active cytokine which plays an important role in the regulation of apoptosis. TNF- $\alpha$  has been shown to be upregulated in MDS by various investigators (Gersuk *et al.*, Br J 1998, Mundle *et al.*, Am J 1999, Molnar *et al.*, 2000, Deeg *et al.*, 2000, Allampallam *et al.*, 2002, Parker 2000). Elevated serum TNF- $\alpha$  values have been found in patients with high-risk MDS and high serum TNF- $\alpha$  concentrations are also predictive of shortened survival in MDS patients (Alexandrakis 1998, Koike 1995).

The aim of the present study was to evaluate the bone marrow PCNA PI and TNF- $\alpha$  serum levels in MDS patients and to question the prognostic significance of PCNA in this group of patients.

## Materials and Methods

### Patients

A total number of 51 patients with primary (de novo) MDS were analysed. The patients were diagnosed between 1996 and 2002 at the University Hospital of Heraklion. The study group included 35 men and 16 women with a median age of 73 years (range 59-91 years). Classification was performed according to the French-American-British (FAB) criteria (Bennett 1982). According to these criteria, the MDS group included 19 patients with refractory anemia (RA), 6 patients with RA with ringed sideroblasts (RARS), 12 patients with RA with excess of blasts (RAEB), 5 RA with excess of blasts in transformation (RAEB-t) patients and 9 patients with chronic myelomonocytic leukemia (CMML). Patients with RA and RARS were grouped as patients with low risk MDS for leukemic transformation whereas patients with RAEB, RAEB-t and CMML as high risk MDS.

In 35 of these patients karyotypic analysis was available and the International Prognostic Scoring System was applied. Eight patients had score 0, 14 had score 1, 6 had score 2 and 7 had score 3 according to the IPSS.

Patients who had previously received chemotherapy or radiation and those with secondary MDS were excluded from the study.

We also studied 15 bone marrow specimens from individuals undergoing biopsies for various diagnostic reasons, but without any abnormality in the bone marrow and the peripheral blood. The median age of the control group was 66 years (range 51-

80 years). Informed consent for the study was obtained from all subjects studied.

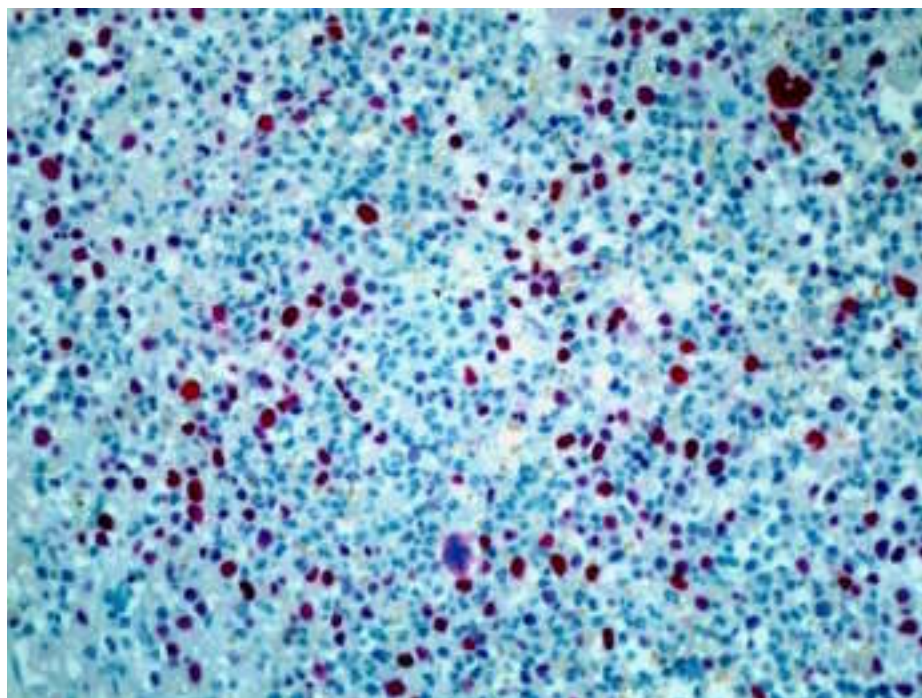
### Bone marrow and blood samples

Bone marrow aspiration and trephine biopsies were performed in all patients from the posterior iliac crest at the time of hospital admission. Diagnosis was made by means of bone marrow aspirate stained with May-Grünwald-Giemsa. The trephine biopsies were fixed in 10% buffered formalin for at least 24 hours, decalcified in 10% EDTA (Titriplex III, M=372.24g/mol, Merck, 64271, Darmstadt, Germany) for 48 hours, and embedded in Paramat extra (BDH Lab Supplies, Poole, BH15 1TD, England). Initially, Hematoxylin-Eosin (H&E) stained 3  $\mu$ m-thick sections were examined. Additional sections were stained with Giemsa, PAS, Gomori for reticulin, Masson trichrome, and Perls; and others were immunostained as routinely in our laboratory, using the immunostaining method described below, with antibodies to CD15, Myeloperoxidase, Glycophorin C, Glycophorin A, CD61, and CD34.

Peripheral venous blood samples were collected at diagnosis. Blood was allowed to clot at room temperature and then centrifuged at 3000 rpm for 10 min. Separated serum was collected, aliquoted and stored at -70°C until further analysis. The concentration of TNF- $\alpha$  was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D systems, Minneapolis, MN, USA).

### Immunohistochemical staining of bone marrow samples for PCNA

Immunostaining was performed using the Super Sensitive (SS) Biotin-Streptavidin Immunodetection System (Biogenex, QA200-OX). Deparaffinized and rehydrated 3  $\mu$ m tissue sections taken on SuperFrost Plus glass slides (O. Kindler GmbH & Co, D-79110 Freiburg, Germany) were heated in a microwave oven in 10 mmol/L citrate buffer, pH 6 at 500 Watts for 5 minutes, three times. After pretreatment for antigen retrieval, the sections were blocked with Power Block Reagent for 20 minutes and then, after washing in TBS, they were incubated with the primary monoclonal antibody to PCNA (DAKO code #M 0879, DAKO DK-2600 Glostrup, Denmark) at a dilution 1/300 for 60 min at room temperature. After washing in TBS, the sections were incubated with Multilink (biotinylated anti-



**Figure 1.** Bone marrow core biopsy from one of the MDS cases included in the study immunostained with anti-PCNA antibody. Immunoreactivity is seen in medullary cells nuclei (red nuclei). LSAB method with Fast Red as chromogen, and counterstaining with Hematoxylin. Original magnification: x100.

immunoglobulins) for 20 minutes, and then they were washed in TBS. The Label (Alkaline Phosphatase-conjugated streptavidin) was applied for 20 minutes and washing in TBS followed. Fast red chromogen with levamisole was applied for 20 minutes. Counterstaining was performed with Papanicolaou la Harris Hematoxylin (Merck KGaA, Darmstadt, Germany) for 30 seconds. For coverslipping the DAKO Glycergel aqueous mounting medium (C-0563, DAKO, Carpinteria, CA, USA) was used. Negative controls by omitting the primary antibody and known positive controls were included in the study.

### Statistical analysis

All results are expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) and the non-parametric Kruskal-Wallis test were used to test for significant differences among the FAB and IPSS subtypes. The non parametric Mann-Whitney tests

were used for high and low risk groups comparisons. The significance of differences of PCNA and TNF- $\alpha$  between the entire group of patients and controls was determined by the Student's t- test.

The Spearman's rank order correlation coefficient was used to determine correlations between the measured parameters. Survival analysis was performed using the product-limit Kaplan-Meier method. Comparisons of survival curves were made using the log-rank test. Values of  $p$  lower than 0.05 were considered as statistically significant.

### Results

The pattern of immunostaining in one of the MDS patients is seen in Figure 1. The PCNA proliferative index was measured as the percentage of positive cells from the total nucleated bone marrow cells. Results for the PCNA determination for the 51 patients samples are shown in Table 1. The mean

**Table 1.** Mean $\pm$ sd of measured parameters in the MDS (according to FAB subtype) and control group.

	RA	RARS	RAEB	RAEB-t	CMML	Controls
PCNA (%)	31.3 $\pm$ 20.6	21.0 $\pm$ 10.2	55.9 $\pm$ 25.2	76.8 $\pm$ 12.7	54.7 $\pm$ 27.5	2.4 $\pm$ 1.7*
TNF- $\alpha$ (pg/mL)	12.9 $\pm$ 6.0	9.7 $\pm$ 5.8	16.6 $\pm$ 5.1	19.5 $\pm$ 6.9	29.9 $\pm$ 12.2	7.2 $\pm$ 2.3*

\* $p$ <0.0001 (t-test between total MDS group and control group). Abbreviations: PCNA: Proliferating cell nuclear antigen, TNF- $\alpha$ : Tumor Necrosis Factor alpha. RA: Refractory Anemia, RARS: Refractory Anemia with Ringed Sideroblasts. RAEB: Refractory Anemia with excess of blasts. RAEB-t: Refractory Anemia with excess of blasts in transformation patients. CMML: Chronic Myelomonocytic Leukemia

**Table 2. Mean±sd of measured parameters in grouped MDS subtypes (low risk: RA and RARS, high risk: RAEB, RAEB-t and CMML).**

	Low risk	High risk
PCNA (%)	28.8±19.0	59.5±24.9*
TNF-α (pg/mL)	12.1±6.0	21.8±10.2*

\* $p < 0.001$  (Mann Whitney test)

**Table 3. Mean±sd of measured parameters in grouped MDS subtypes according to IPSS.**

IPSS score	Number of patients	PCNA (%)*	TNF-α (pg/ml)°
0	8	30.12±21.36	12.58±6.38
1	14	38.42±26.78	14.55±8.81
2	6	52.16±25.41	25.71±15.84
3	7	67.28±23.32	20.38±6.13
Controls	15	2.40±1.76^	7.20±2.31^

\*Patients with score 0 and 1 had significantly lower values of PCNA than those with score 3 ( $p < 0.01$ ). °Patients with scores 0 had significantly lower values for TNF-α than those with scores 2 and 3 ( $p < 0.05$ ). ^PCNA and TNF-α significantly higher in MDS patients in comparison to controls ( $p < 0.001$ ).

levels of proliferation as measured by PCNA in our 51 MDS patients was  $44.5 \pm 26.9$  and thus higher compared to the mean PCNA level ( $2.4 \pm 1.7$ ) found in the control group ( $p < 0.0001$ ). Statistically significant differences in PCNA expression were found, with highest values detectable in RAEB-t, RAEB and CMML FAB subtypes ( $p < 0.001$ ) and the IPSS=3 group ( $p < 0.01$ ). Significant differences were found also when MDS patients were divided into the two risk groups. The highest values were found in the high-risk group in comparison to the low-risk ( $p < 0.001$ ) (Table 2).

In the entire group of patients serum TNF-α concentrations (mean±SD:  $17.1 \pm 9.6$  pg/mL) were significantly higher than the respective ones of controls (mean±SD:  $7.2 \pm 2.3$  pg/mL,  $p < 0.0001$ ). Significant differences were also found in TNF-α levels among the MDS subtypes ( $p < 0.001$ ). Elevated serum TNF-α levels were seen in patients of the high-risk MDS groups CMML, RAEB-t, RAEB in comparison to the low risk-group RA and RARS ( $p < 0.001$ ). TNF-α was significantly elevated in the IPSS scores 2 and 3.

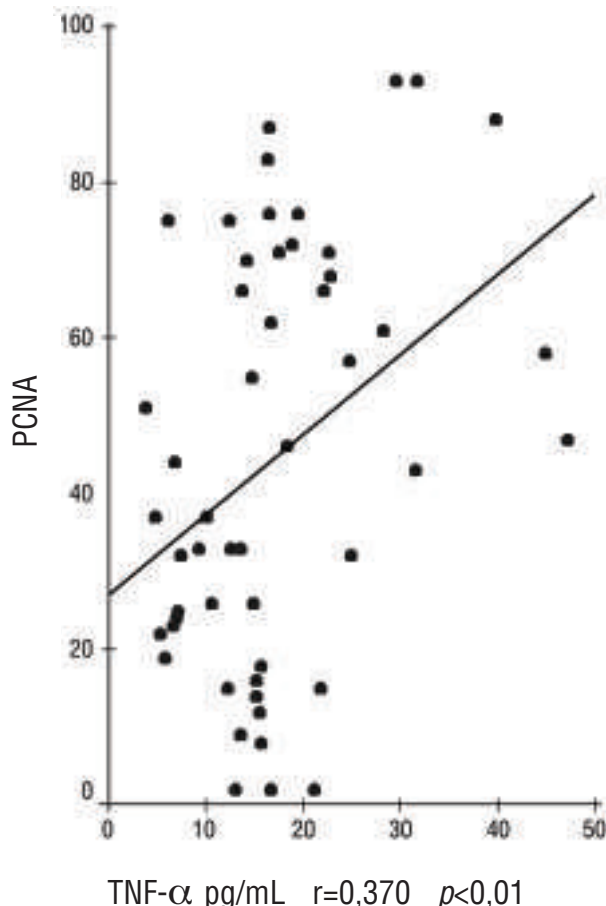
A statistically significant positive correlation was noted between TNF-α values and PCNA PI expression of the nucleated cells of the bone marrow ( $r = 0.37$ ,  $p < 0.008$ ) (Figure 2).

We used a cut-off point of 43 that corresponds to the median level of PCNA in order to define prog-

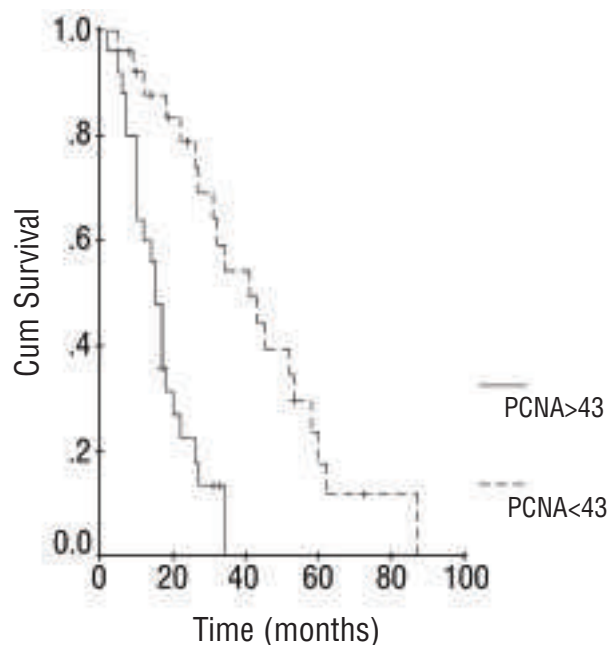
nostic subgroups of patients. For both MDS groups, patients with PCNA levels higher than 43 had significantly lower survival times than patients with PCNA levels less than 43 ( $p < 0.001$ ) (Figure 3).

## Discussion

PCNA has been identified as an auxiliary protein of DNA polymerase delta and is synthesized in correlation with the proliferative state of the cell (Bravo 1986, Celis 1987, Raza 1997). Our results showed an increased proportion of PCNA-positive cells in the bone marrow of MDS patients compared with control cases. This difference was more pronounced than that reported in a previous study but this may be due to the different percentages of the MDS subgroups (Kitagawa 1993). Furthermore, PCNA has been shown to be helpful in the differentiation of aplastic anemia from hypoplastic MDS as it is found increased in the second case. (Orazi 1997)



**Figure 2. Correlation between PCNA proliferation index and serum TNF-α in MDS patients.**



**Figure 3.** Kaplan-Meier survival plot in 2 MDS groups with PCNA levels above and lower than the median (43%). The median survival time for the high PCNA group was  $15 \pm 1$  months whereas for the low PCNA group was  $41 \pm 8$  months ( $p < 0.001$ ).

In our study, proliferative activity (PCNA PI) showed significant differences between low-risk disease and high-risk MDS, with the highest levels in patients with refractory anemia with excess of blasts in transformation. According to cell kinetic studies, a prevalent cell production rate is detectable in the myeloblastic compartment and this *in vitro* finding is reflected by a significant increase in the PCNA labeling index (Allen 1993, Thiele 1996, Thiele 1997). The accumulation of the PCNA mRNA in cells followed by the synthesis of high levels of the protein is stimulated by growth factors, and as reported in a previous study, PCNA levels in leukemia reflect the activity of other growth regulatory genes that are associated with cell proliferation (Keim 1990, Kitagawa 1993).

This increase of proliferative activity is in contrast to the peripheral cytopenias observed in MDS. The mechanism of haematopoietic failure in patients with MDS is not clear. It has been hypothesized that a lack of haematopoietic growth factors and the abnormal expression of negative regulators of haematopoiesis such as TNF- $\alpha$  may be responsible (Greenberg 1992, Maciejewski 1995). In our patients, elevated serum levels of TNF- $\alpha$  were found in high-risk patients as compared with low-risk. TNF- $\alpha$  plays an important role in the regulation of

apoptotic cell death and is upregulated in MDS (Gersuk 1998, Mundle 1999, Molnar 2000, Deeg 2000). It has been proposed that increased apoptosis in MDS serves as a counterbalance for the increased proliferation rate in order to sustain cell numbers (Parker 2000). This is in agreement to the finding of a positive correlation between PCNA activity and TNF- $\alpha$  in our patients.

In the present study, we also showed that an increase of proliferative activity higher than the median, which was calculated at approximately 40%, was associated with a worse prognosis. Whereas this seems logical as PCNA is increased in the MDS subtypes with increased blast numbers, which display a worse prognosis, in a previous study PCNA was not correlated with the percentage of CD34 positive cells. Furthermore, PCNA in MDS was not different from AML (Lin 2002). This implies that the prognosis does not depend solely on the blast number but on the proliferative potential and the effect of apoptosis, which is not reflected in the FAB classification. In another study, although TNF- $\alpha$  bone marrow levels in MDS were elevated in comparison to samples from normal marrow donors, there was no correlation with FAB subtype or the cytogenetic risk group (Deeg 2000). Furthermore a substantial percentage of MDS patients die due to the refractory cytopenias (infections, hemorrhage) without entering a leukemic phase. Therefore the combination of ineffective hematopoiesis mediated via molecules such as TNF- $\alpha$  (Stasi 1997, Rosenfeld 2002,) and enhanced proliferation as assessed by PCNA is most likely a better determinant of survival in MDS patients.

In conclusion, our study demonstrated that high bone marrow PCNA levels are associated with a shortened survival in MDS and that high proliferative activity is associated with increased serum TNF- $\alpha$ . Taken together, PCNA PI might be considered as an additional prognostic factor in this group of patients.

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