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Prognostic significance of morphological assessment of plasma cells in multiple myeloma

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Multiple myeloma (MM) is a hematological malignancy caused by clonal proliferation of malignant plasma cells (PC). The aim of the work is to determine prognostic significance of morphological subtypes of PC in relation to overall treatment response, long-term survival and other conventional prognostic parameters. One hundred and thirty-nine newly diagnosed MM patients who underwent autologous transplantation in clinical trials conducted in one center were included. Percentual representation of subtypes of plasma cells in bone marrow was measured based on progressive analysis of nucleolus, nuclear chromatin and ratio of nuclei to the volume of cytoplasm (N/C ratio) creating 8 subtypes P000-P111 and four subclassifications of cells. Mature plasma cells (P000, P001) were found in 42.4% of patients; proplasmocytes I (P010, P011, P100) in 38.1% of patients, and proplasmocytes II (P101, P110) in 19.4% of patients. Patients who reached treatment response after autologous transplantation had statistically significant lower frequency of mature plasma cells than patients with no treatment response (median 24.0% vs. 36.0 %; p=0.032). Patients with mature plasma cells of subtype P000 < 10% had significantly shorter overall survival than patients with value P000 ≥ 37% (median 46.8 months vs. 77.8 months; p = 0.020). Patients with proplasmocytes II subtype P110 < 3% had longer time to progression than patients with subtype P110 ≥ 31% (median 54.6 months vs. 22.4 months; p=0.045). Our work brings valuable prognostic information and correlation with other prognostic factors as well as total treatment response and survival in MM patients who underwent autologous transplantation.

Key words: multiple myeloma, morphology, plasma cells, cytogenetics, prognostic significance, nucleus

Multiple myeloma (MM) is the second most common hematooncological disease caused by malignant clonal proliferation of cells of the B-cell lineage. Morphological evaluation or rather numerical assessment of plasma cells (PC) in bone marrow (BM) is a basic diagnostic criterion even in time of modern genomic analyses [1, 2, 3, 4].

Our analysis is based on evaluation of three criteria- presence of nucleoli, character of nuclear chromatin and ratio of nuclei to the volume of cytoplasm. Prognostic significance of such an evaluation was originally proven in the era of conventional chemotherapy. It is based on the presence of immature plasmoblasts denominated as subtype P111 [5, 6, 7]. Work by Goasguen et al. [5] showed the influence of plasmoblast presence on overall survival in MM patients treated by standard treatments. Overall survival (OS) was found to be 54 months for patients with plasmoblasts < 2% in comparison to 20 months for patients with plasmoblasts ≥ 2% [5]. In the largest morphological study conducted in Japan on 1119 patients, the presence of more mature plasmocytes also showed better prognosis [8].

In our morphological analysis of plasma cells of patients undergoing autologous transplantation, the plasmocytes were divided into 4 types and 8 subtypes based on cell maturity – similarly to the French study [5, 9].

The aim of this study was to provide prognostic significance of above mentioned subtypes of PC in connection to overall treatment response, overall survival and to define the relationship to other conventional prognostic parameters. It is the first analysis *in extenso* for a homogenous set of patients treated by autologous transplantation.

Materials and methods

Characterization of sample group.

One hundred and thirty-nine newly diagnosed MM patients who underwent autologous transplantation at the Department of Internal Medicine-Hematooncology in FN Brno were enrolled in this study; these patients were treated between 1996 and 2007 in clinical trials 4W and CMG2002. All patients signed informed consent form approved by the Ethical Committee of the hospital.

All patients underwent peripheral blood sampling to follow albumin, beta-2-microglobulin, CRP, LDH, calcium, hemoglobin, thrombocytes. Samples of BM were obtained by sternal puncture or aspirate of trephine biopsy.

The protocols of these two clinical studies had the same induction treatment (4x VAD; vincristine, Adriamycin and Dexamethazon), myeloablative treatment using 200mg/m^2 melphalan followed by support of hematopoietic cells (in this text, this is abbreviated as autologous transplantation). The studies differed in consolidation or maintenance treatment that had no significant influence on the outcome of studies [10, 11, 12].

The sample group was composed of 62 females (44.6%; 62/139) and 77 males (55.4%; 77/139), median age was 55 years (SD = 7.1). Demographic data are presented in Table 1.

Morphological evaluation.

Cell maturity was evaluated based on published methodology [5]. Analysis of 8 subtypes (P000-P111) showed 4 types of plasmocytes of different maturity: mature plasmocytes (P000 and P001), proplasmocytes I (P010, P011 and P100), proplasmocytes II (P101 and P110) and plasmoblasts (P111) (Fig.1).

Each BM smear was evaluated and scored after fixation in May-Grunwald and stained using Giemsa-Romanovsky (Pappenheim panoptic staining method) [13, 14]. To confirm MM diagnosis, we used the score of ≥10% of PC out of all nucleated blood elements in the smear. To evaluate the BM smear, we used microscope Olympus BH-2, objectives 100x, 20x, 10x and hematological counter SH-96/24D. For each patient, 100 PC were evaluated based on 3 criteria in the following order:

1. Nucleolus – presence of nucleolus was designated 1, absence 0
2. Chromatin – presence of blastic chromatin 1, presence of condensed chromatin 0
3. Ratio of nucleus to cytoplasm volume (N/C ratio) – size of nucleus to the volume of cytoplasm. Ratio N/C > 0.6 is designated 1, ratio N/C < 0.6 is 0.

Out of 139 patients analysed in this study, autologous transplantation was not done for 9.4% (13/139) – five patients died after induction VAD therapy, six patients were removed from preparation for autologous transplantation for other reasons after VAD induction, and one patient died after stimulation chemotherapy. One patient died shortly after autologous transplantation.

Evaluation of cIg-FISH. For detection of chromosomal abnormalities, immunofluorescent method of light chain staining of cytoplasmic immunoglobulins together with I-FISH (cIg-FISH) was done as previously published [15] with slight changes to the protocol [16]. The presence of del(13q14) and 17p13, amplification of 1q21, presence of translocation of the IgH locus and translocations t(4;14) and t(11;14) were analysed in PC. For I-FISH, the following probes were used : LSI 13 (RB1) Spectrum Orange Probe, LSI p53 (17p13.1) Spectrum Orange Probe, LSI IGHC/IGHV Dual Color Probe, LSI IGH/FGFR3 Dual Color Probe, LSI IGH/CCND1 Dual Color Probe and reference probes LSI 13q34 Spectrum Green a CEP 17 Alpha Spectrum Green (Abbott Molecular). For 1q21 amplification, fluorescently marked clone BAC DNA

Table 1. Demographic data of patients, clinical classification of MM based on Durie-Salmon classification (D-S stage), A-B subclassification and international prognostic index for MM (ISS) according to IMWG 2006.

Data set characterisation			
Average age (SD)		55 (7.1)	Percentage
Number of patients 139			
Sex			
	Female	62	44.6
	Male	77	55.4
Subtypes of plasma cells in MM patients			
Plasmocytes (P000 a P001)		59	42.4
Proplasmocytes I (P010, P011 a P100)		53	38.1
Proplasmocytes II (P101 a P110)		27	19.4
Plasmablasts (P111)		0	0
Type of paraprotein			
	IgG	81	58.3
	IGA	43	30.9
	B-J	11	7.9
	IgM	2	1.4
	IgD	1	0.7
	IgG + IgA	1	0.7
Durie and Salmon stage			
	I	12	8.6
	II	29	20.9
	III	98	70.5
A-B stage			
	A	122	87.8
	B	17	12.2
ISS stage N = 104			
	1	48	46
	2	38	37
	3	18	17
Patients with autologous transplantation		126	90.6
Patients excluded from study		13	9.4
1	Death after VAD induction	5	3.6
2	Other reason for induction end	6	4.3
3	Excluded after stimulation	1	0.7
4	Death after transplantation	1	0.7

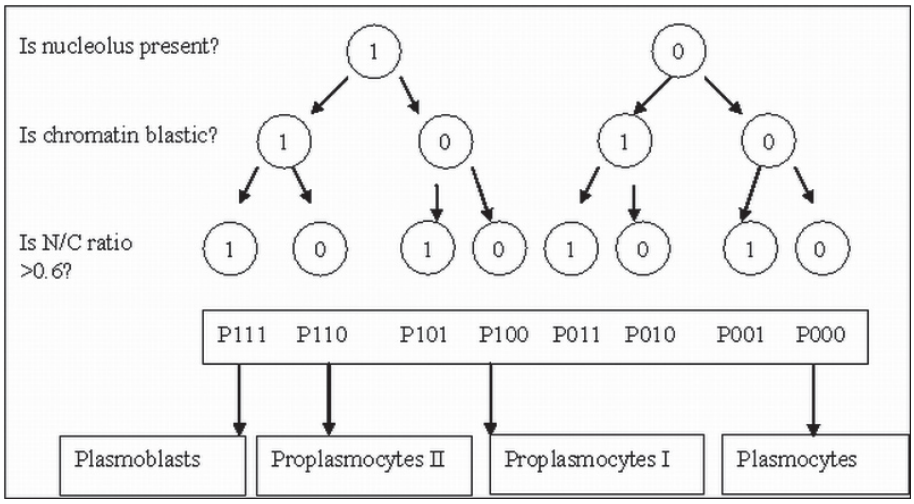


Figure 1. Algorithm for automatic subclassification of plasma cells in MM. There are three questions that need to be answered-answers are either yes (1) or no (0). This algorithm is based on Goasguen et al, 1999 [5].

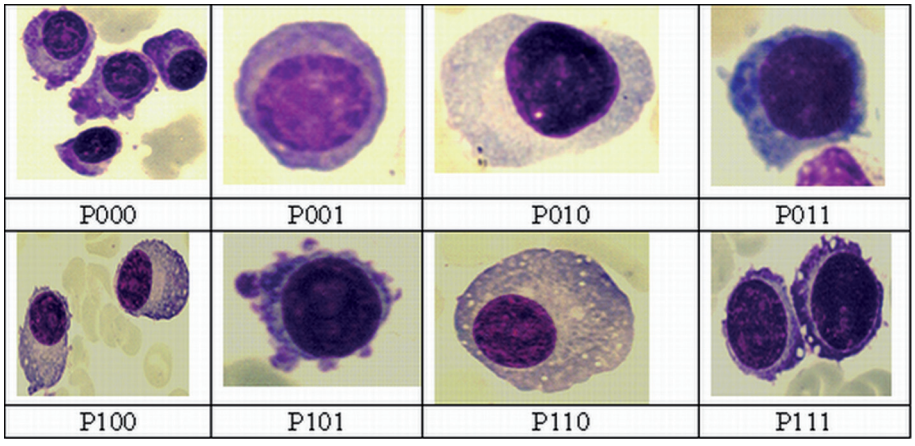


Figure 2. Morphological differences of PC subtypes.

RP11-205M9 was used. For each patient, 50 to 100 PC were evaluated. Cut-off values recommended by the European Myeloma Network [17] were used: for deletions and numerical aberrations, the cut-off level was set at 20%; for translocations in IgH locus as well as other translocations, the cut-off level was set at 10%. Fluorescent microscope Olympus BX 61 was used. The picture was scanned by a CCD camera Vosskuhler 1300D and analyzed by LUCIA-KARYO/FISH/CGH software (Laboratory Imaging, Prague, Czech Rep.).

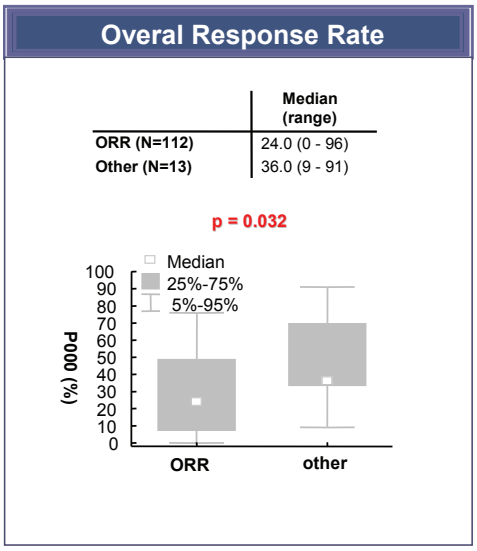
Statistical analysis of prognostic significance of PC morphology. Statistical analysis was performed using STATISTICA 8.0 StatSoft, Inc. (2007) program. For P000-P111 parameters, normality was not proven, so non-parametric tests were used. Relationship to laboratory values of albumin, beta-2-microglobulin, CRP, LDH, calcium, hemoglobin, thrombocytes was evaluated using Spearman's correlation coefficient (Rs). The relationship of P000-P111 parameters with categorical parameters (treatment response, type of paraprotein, D-S and ISS stages, cytogenetic abnormality) was evaluated by Mann-Whitney test; to show statistical significance of P000-P111 with categorical parameters (ISS and Durie-Salmon stages), Kruskal-Wallis Anova Test was performed. For survival analysis, median of survival was calculated using Kaplan-Meier curves, and differences between groups were tested by Log-rank test. All tests were performed at 5% level of significance.

Results

Prognostic significance analysis of PC morphology. Morphological assessment for 139 newly diagnosed MM patients (demographic data are presented in Table 1) was performed

as follows: in 42.4% of patients (59/139) there were mature plasmocytes (P000, P001), in 38.1% of patients (53/139) there were proplasmocytes I (P010, P011, P100), in 19.4% of patients (27/139) there were proplasmocytes II (P101, P110) and plasmoblasts (P111) were found in 0 % of patients (for algorithm of morphology see Fig. 1). Morphological differences among various stages of plasma cells are presented in Fig. 2

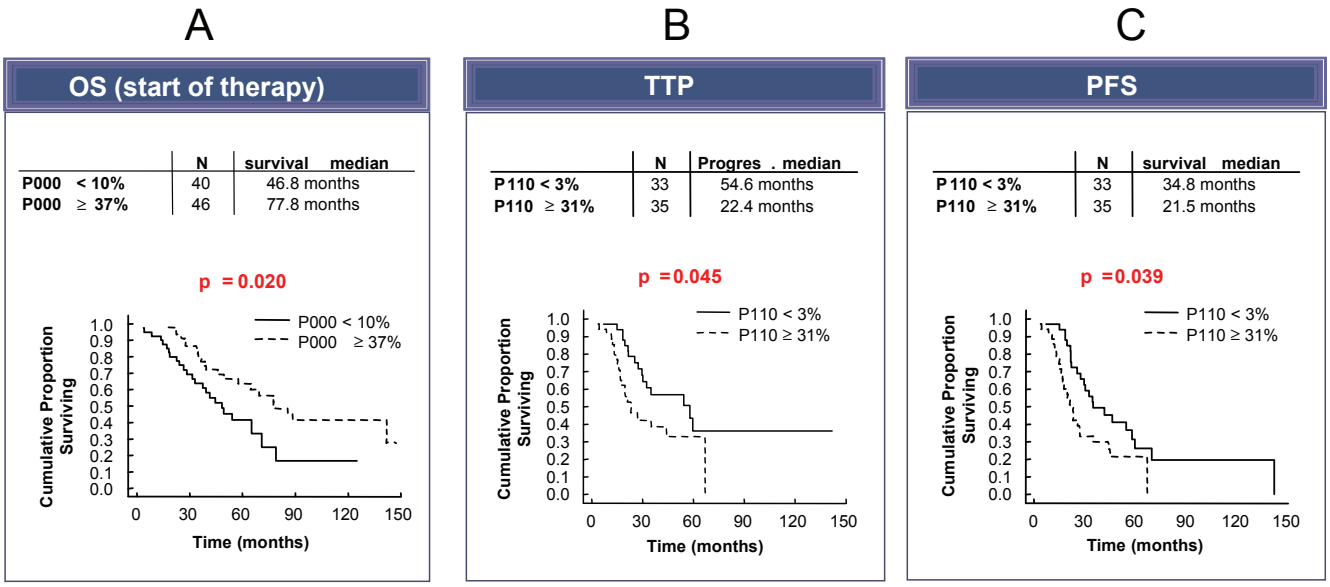
Correlation of PC subtypes with overall response rate. Overall response rate (ORR) was evaluated before and after transplantation. There was no statistically significant relationship between PC subtypes and ORR before transplantation. However, a statistically significant correlation between subtype of mature plasmocytes and ORR in patients who underwent BM transplantation was found. A group of patients with ORR had a lower number of mature plasmocytes than the group of patients without treatment response (median 24.0% vs. 36.0 %; p= 0.032). We noticed a trend towards a higher number of proplasmocytes II in group of patients with treatment response in comparison with the group without treatment response (median 10.5% vs.4.0%; p = 0.061). Results are shown in Graph 1.



Graph 1. Representation of statistical significance of relationship between P000 subtypes and treatment response after autologous transplantation.

Correlation of overall survival, time to progression and survival without progression with PC subtypes. After PC division into groups based on median and mean, there was no significant difference among groups of patients when subtypes and overall survival were compared. The same was true when patients were divided into terciles and quartiles. That is why only outer groups were compared for quartiles (values < 25% percentile vs. values of ≥ 75% percentiles) and terciles (values < 33.3% of percentiles vs. values ≥ 66.7% percentiles).

Overall survival. When comparing outer groups of terciles of plasmocytes subtypes, a statistically significant correlation of mature plasmocytes with overall survival (OS) was found. The group of patients with mature plasmocytes < 10% (33.3% percentile) had a shorter OS than the group of patients with mature plasmocytes ≥ 37% (33.3% percentile) (median 46.8 months vs. 77.8 months; p = 0.020). We have also noticed a numerically significant trend to longer OS for patients with proplasmocytes II < 3% (25% percentile) when compared to patients with proplasmocytes II ≥ 31% (33.3% percentile) (median 93.2 months vs. 53.6 months; p = 0.073).



Graph 2 Analysis of overall survival (OS) in subtypes P000 (A), and PFS and TTP in subtype P110 (B,C) .

Time to progression and progression-free survival. Significantly longer time to progression (TTP) was found for group of patients with proplasmocytes II < 3% in comparison to group of patients with proplasmocytes II ≥ 31% (median 54.6 months vs. 22.4 months; p= 0.045). For progression-free survival (PFS), similarly to TTP, a group of patients with proplasmocytes II < 3% had a longer time to progression than the group of patients with proplasmocytes II ≥ 31% (median 34.8 months vs. 21.5 months; p=0.039). Results are presented in Graph 2.

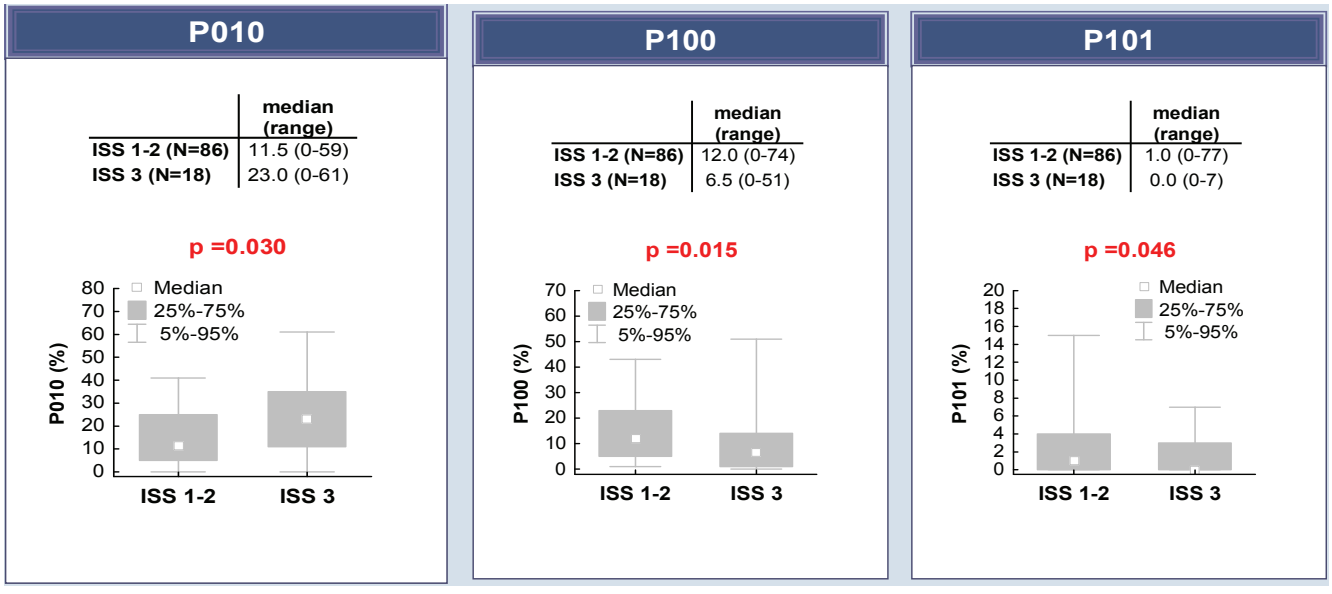
Correlation of PC subtypes with conventional prognostic factors. Statistically significant relationship was found for proplasmocytes I - a positive correlation with albumin (Rs=0.229; p=0.007) and negative correlation with CRP (Rs = -0,194; p=0,027), and also for patients with mature plasmocytes where a negative correlation with LDH (Rs = -0,196; p= 0,024) was observed. Although these differences were statistically significant, it was a weak correlation. Group of patients with IgG had a lower number of proplasmocytes II than group of patients with other types of paraprotein (median 9.0% vs. 15.0%; p=0.037). Another group with IgA had a higher number of proplasmocytes I than groups with other types of paraprotein (median 17.0 % vs. 9.0%; p=0.025). We found that patients with B-J had some plasmoblasts when compared to patients with other types of paraprotein, although this number was still very small (median 5.0% vs. 1.0%, p=0.028). In group of patients with IgG, a trend was noticed toward a higher number of mature plasmocytes when compared to groups of patients with other types of paraprotein (median 31.5% vs. 17.0%; p=0.060).

There was no statistically significant correlation between subtypes of PC and clinical stages based on Durie-Salmon (D-S) classification, not even to substages A-B. However, a trend to a difference in the number of proplasmocytes II based on D-S was observed (median 5.5 % vs. 5.0 % vs. 13 %; p=0.066). A lower number of proplasmocytes I in patients in ISS stages I and II when compared to patients in stage III of ISS (median 11.5% vs. 23.0%; p=0,030) was proven; this is in contrast to proplasmocytes I and II where a higher number of patients in stages I and II of ISS when compared to stage III patients was noticed (median 12.0% vs. 6.5%; p=0,015 for proplasmocytes I and 1.0% vs. 0.0%; p=0.046 for proplasmocytes II. Results are in Graph 3.

Correlation of PC subtypes with cytogenetic abnormalities detected by cIg-FISH. Patients without del(13q14) had a higher frequency of mature plasmocytes than patients with del(13q14) (median 35.0% vs. 13.0%; p=0.014). On the other hand, patients with del(13q14) had a higher value of proplasmocytes II than patients without del(13q14) (median 36.5% vs. 6.0%; p=0.012). This difference was nearing statistical significance. For del(14q32), t(11;14), t(4;14), del(17p13) and gain of 1q21, no significant difference in frequency of morphological classification of the PC subtypes P000 – P111 was found in patients with or without chromosomal aberrations.

Discussion

Since 1984, it has been known that the presence of immature PC (plasmoblasts) is correlated with worse prognosis in MM. PC of these patients were divided into 4 subtypes (mature, intermediate, immature and plasmoblasts). The most extensive clinical study from that time period is the Japanese study of 1119 patients published in 1994 [8]. Patients who survived



Graph 3. Comparison of relation between parameters (P010, P100, P101) and ISS stage.

for more than 10 years had more mature plasmocytes in BM biopsies when compared to patients with shorter survival (54% vs. 31%; p= 0,01). In 1999, there was a study published categorizing maturity of plasmocytes based on 3 criteria – presence of nucleolus, maturity of chromatin, ratio of nucleus volume to volume of cytoplasm N/C [5]. The results of analysis of eight subtypes P000 – P111 are four types of plasmocytes of different maturity: plasmocytes, proplasmocytes I, proplasmocytes II and plasmoblasts; their prognostic significance has been evaluated during the era of conventional chemotherapy. In total, 90 MM patients were included in the study; out of these patients, 59 patients with plasmoblasts (subtype P111) < 2% had longer survival when compared to patients with P111 ≥2% (median survival 54 vs. 20 months; $p=10^{-4}$) [5].

An interesting but not very surprising fact was the relationship between morphology of plasmocytes to treatment response – patients who reached partial remission had a lower number of mature plasmocytes than patients without treatment response (median 24.0% vs.36.0%; p=0,032). A reverse trend was true for immature forms. Although this result turned out to be statistically significant, it is of no importance for clinical use. Generally, it seems to be true that immature forms represent cells with intensive metabolism that are more sensitive to outside influence. However, if chemotherapy does not completely destroy these cells, their renewal and dynamics is faster. This explanation seems to correspond to results of overall survival – patients with mature plasmocytes <10% had shorter overall survival than patients with mature plasmocytes ≥ 37%. Difference of 31 months (median 46.8 months vs. 77.8 months; p = 0.020) is significant even clinically. Similarly, difference of 40 months is clinically significant for the reverse trend for immature forms- proplasmocytes II (median 93.2 months for proplasmocytes II < 3% vs. 53 months for proplasmocytes II≥ 31%; p = 0.073). This corresponded to similar results of PFS and TTP (median 54.6 months for proplasmocytes II <3% vs. 22.4 months for proplasmocytes II ≥ 31%; p= 0.045), where difference of 32 months was clinically significant. It is obvious that more detailed morphological evaluation still brings very cheap, quick and significant prognostic information. It will be interesting to analyze further the correlation between reaching complete remission and maintaining complete remission in correlation to entry maturity of plasmocytes.

From the clinical point of view, it is necessary to establish a clinically significant cut-off values. We did not find any significant differences in overall survival when patients were divided by median and mean as well as quartiles and terciles – that is why we compared marginal groups into quartiles and terciles. When cut-off values are set correctly, the results were prognostically significant not only statistically but also clinically since the difference was 31-40 months.

When correlating subtypes of PC with other conventional prognostic factors, corresponding values were generally found. A trend toward increase of number of proplasmocytes II in patients of D-S stage III in comparison to patients of stages D-S I and II (median 13.0% vs. 5.5%, 5.0%; p=0.066). A lower number of proplasmocytes I in patients of ISS stages I and II when compared to ISS stage III patients was found (median 11.5% vs. 23.0%; p=0.030), in contrast to proplasmocytes I and proplasmocytes II where a higher number of patients in ISS stages I and II in contrast to patients in stage II of ISS (12.0% vs. 6.5%; p=0.015 for proplasmocytes I, and 1.0% vs. 0.0%; p=0.046 for proplasmocytes II.

We tried to correlate cytogenetic abnormalities detected by cIg-FISH. More precise analysis was possible only for del(13q14) – this is connected to the number of patients in this group and usual uniform distribution of this probe positivity in followed groups. A higher number of mature plasmocytes in patients without del(13q14) (median 35.0% vs. 13.0%; p=0.014) was found; on the other hand, a higher number of proplasmocytes II in patients with del(13q14) (median 36.5% vs. 6.0%; p=0.012). For translocations t(14q32), t(11;14), t(4;14), del (17p13) and gain 1q21, no statistically significant difference of cell number P000-P111 in patients with/without chromosomal aberration was found. However, we cannot conclude anything as the number of evaluated patients was limited due to frequency of probe positivity in the case of IgH translocation and del(17p13) and the fact that evaluation of gain1q21 has been performed in our lab only since 2005 [16].

Morphological evaluation or rather numerical assessment of plasma cells in bone marrow still remains a basic diagnostic criterion for MM even during times of genomic, proteomic and cytomic analyses. It is also a method available in all parts of the world. More precise but also more personnel, time and economically less consuming morphological evaluation of 8 subtypes of plasma cells in bone marrow brings significant prognostic information. This information is necessary for treatment protocols using conventional chemotherapy and autologous transplantation. If it is valid even in the era of immunomodulatory drugs and proteasome inhibitors remains to be seen.

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