Prognostic significance of plasma cell propidium iodide and annexin-V indices and their mutual ratio in multiple myeloma*

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The aim of this study was a contemporaneous measurement of plasma cells proliferative and apoptotic activity in patients examined at the time of multiple myeloma (MM) diagnosis before initiation of chemotherapy, focussed on the following aspects: determination of prognostic significance of plasma cell propidium iodide (PC-PI) and annexin-V FITC (PC-AI) indices; optimal cut off of PC-PI and PC-AI with regard to overall survival; calculation of summary kinetic index of plasma cells (PC-PI/AI ratio) for evaluation of its prognostic importance; determination of an index (out of PC-PI, PC-AI and PC-PI/AI) showing the closest relation to prognosis of multiple myeloma.

The analyzed 122 patients fulfilling SWOG multiple myeloma criteria were treated by conventional chemotherapy. Plasma cell proliferative activity was measured by means of PC-PI examined by flow cytometry using a DNA/CD_{138} double staining technique. For detection of plasma cells entering apoptosis (PC-AI), flow-cytometry method with annexin-V FITC and MoAb CD_{138} was used.

The PC-PI median in 122 patients was 2.6(0.4–4.8)%. The sequence prognostic analysis showed that the optimal PC-PI cut off was 2.9% and displayed a significant relationship with overall survival (OS) (p=0.031). The group of 94 patients had PC-AI median of 5.0(1.4–24.5)%. The best statistical significance of the rate of apoptosis related to overall survival was found at cut off value of 4.4% (p=0.022). The median of overall kinetic index of plasma cells (PC-PI/AI) examined in 94 MM patients was 0.5(0.05–2.60) and the overall kinetic index was found to display a very good relationship to OS at the cut off value of 0.71 (p=0.032). All the three indices expressing various aspects of kinetics of plasma cells allow the stratification of patients into two prognostically different groups with statistically significantly different medians of overall survival: good risk – OS still undeterminable at the time of analysis; bad risk – M: OS was for PC-PI 17 months, for PC-AI 23 months and for PC-PI/AI 16 months. The ratio of both indices, i.e. PC-PI/AI, however did not bring any further contribution to overall survival/prognosis evaluation, when compared with single PC-PI and PC-AI.

Results of present study indicates that the evaluation of both proliferation and apoptotic activities of plasma cells is important for prognosis thus extending possibilities of initial stratification of MM patients into groups with different prognostic risk.

Key words: multiple myeloma, plasma cell, propidium iodide index, annexin - V index, overall kinetic index of plasma cells, apoptosis, prognosis

Multiple myeloma (MM) is a clonal lymphoproliferative disease that affects terminally differentiated B-cells, i.e. plasmocytes, which typically display a variable proliferative activity and different resistance to apoptosis with latent accumulation of myeloma cells in the bone marrow [1, 2]. The disharmony of proliferation and down-regulation or disruption of apoptosis is considered as one of crucial mechanisms of myeloma cell expansion [3]. The expansion of myeloma cell neoplastic clone is determined by an equilibrium between proliferation and induction or blockade of apoptosis. It seems that in the pathogenesis of MM the key event is not only the loss of myeloma plasma cells growth control, but also the inhibition of their apoptosis. While significant im-

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portance of proliferative properties of plasma cells (PC-PI) for MM prognosis has been confirmed [4–8], only a few clinical studies reported on evaluation of myeloma cells apoptotic intensity [9, 10]. It is even striking, that despite numerous papers dealing with apoptosis in MM, most studies are of experimental character, focussed mostly on plasma cell apoptosis under *in vitro* conditions in constrast with sporadical reports oriented to clinical situation.

The aim of this study was to measure contemporaneously the plasma cell proliferative (PC-PI) and apoptotic (PC-AI) activities in patients analyzed at the time of MM diagnosis with the following purposes:

a) Determination whether not only the evaluation of plasma cell proliferation activity using propidium iodide index (PC-PI), but also examination of apoptosis rate evaluated by means of annexin-V FITC index (PC-AI) could be of prognostic significance;

b) Determination of optimal cut off value of propidium iodide (PC-PI) and annexin-V (PC-AI) indices for evaluation of overall survival;

c) Calculation of overall kinetic index of plasma cells (PC-PI/AI ratio) with respect to its prognostic importance;

d) Determination which of the three indices, i.e. propidium--iodide proliferation index (PC-PI), annexin-V apoptotic index (PC-AI) or overall kinetic index of plasma cells (PC-PI/AI) has the closest relation to prognosis.

Patients and methods

The analyzed group of 122 patients fulfilling SWOG multiple myeloma diagnostic criteria [11] was examined at the time of MM diagnosis before initiation of the therapy during 1994–2004, while in the group with examination of PC-AI were 94 patients during 1997–2004. Serum electrophoresis and immunofixation method, performed in all patients, identified following monoclonal immunoglobulins: IgG 66%; IgA 22%; light chain only 10% and IgM 1%. Of the serum monoclonal proteins, 68% were kappa, 31% were lambda. One percent of the patients had no monoclonal protein identifiable in the serum and/or in the urine. The median age at diagnosis in this series was 66 (44–85) years, (male-to-female ratio 0.8: 1.0). Clinical stage by the DURIE-SALMON system [12] was distributed as follows: I – 15(12%); II – 52(43%); III – 55(45%); substage B – 32(26%). In the examined group of MM patients, a systematic conventional chemotherapy, i.e. VBMCP (M_2-protocol), VAD, Cy-VAD, CIDex, and sometimes MP regimens were used.

Plasma cell proliferative activity (proportion of S-phase plasma cells) was measured by means of propidium iodide index (PC-PI) by flow cytometry (Coulter Epices XL Coulter Corporation) using a DNA/CD_{138} double staining technique allowing to evaluate the fluorescence of intercalated propidium iodide that is proportional to cellular DNA content, while plasma cells were identified by MoAb against syndecan-1 (CD_{138}) [13]. PC-PI was examined in bone marrow aspirates after separation of cells using a density gradient (Ficoll Verografin). Special Multicycle software for each sample analysis was used to evaluate plasma cells present in the S-phase of the cell cycle (Multicycle for Windows ver. 3.0 Phoenix Flow Systems) [14–16].

Apoptotic properties of plasma cells were examined in the bone marrow aspirate placed in heparinized RPMI 1640 medium, followed by cell separation using a density gradient and identification of myeloma plasma cells with MoAb anti-CD_{138} (CD_{138} PE, Biotech), which is plasma cells specific. To detect phosphatidyl serine expression on the surface of apoptotic cells, the Annexin-V FITC kit (Immunotech, Marseille, France) was used, allowing selective differentiation of the apoptotic, necrotic and secondary necrotic cell population using flow cytometer (Epics XL, Coulter Corporation, Miami, USA) analysis. The apoptotic index was defined as percentual presence of propidium iodide-negative cells showing a specific bound on annexin-V [9, 17].

The Kaplan-Meier product limit method was used to obtain the survival curve; log rank test ($p<0.05$) was employed to determine the overall survival time differences.

Results

It was found that the PC-PI median in the 122 MM group was 2.6 (0.4–4.8)%. Results of individual steps of the performed analysis are given in Table 1. The sequence prognostic analysis showed that in case of evaluation of proliferation PC-PI index, cut off value of 2.9% divided MM patients into two prognostically different groups, where a median of OS of patients with a high value of PC-PI index ≥2.9% was only 17 months, while in the group of patients with a low value, i.e. <2.9% remained unidentifiable due to survival of patients at the time of analysis (Tab. 1, Fig. 1). Differences in prognosis between the two compared groups were statistically significant.

In 94 examined MM patients, PC -AI median was 5.0 (1.4–24.5)%. Prognostic analysis of initial values of plasma cells apoptosis rate using PC-AI also proved a high significance of this marker. Unfavourable prognosis of short survival rate was confirmed in patients with a low PC-AI value, i.e. ≤4.4% with overall survival of only 23 months, while in the group of patients with a high value of PC-AI >4.4% the OS median could not be identified at the time of analysis yet (Tab. 1, Fig. 2). In order to enhance sensitivity of overall prognostic estimation, we tried to evaluate the importance of overall index of proliferation and apoptosis of plasma cells calculated as a ratio of propidium iodide and annexin-V indices (PC-PI/AI). Median of overall index examined in the group of 94 MM patients was 0.5 (0.05–2.60). Sequence prognostic analysis showed the highest prognostic potential of the overall kinetic index of plasma cells 0.71. The group of patients with summary index value ≥0.71 was found to have a considerably worse prognosis with median survival of only 16 months, while patients with the index value <0.71 had a

Table 1. Characteristics of the analyzed groups and results of prognostic significance of proliferative and apoptosis properties of the plasma cells and the proliferative/apoptosis plasma ratio in the group of multiple myeloma patients examined at the time of myeloma diagnosis before starting of conventional chemotherapy

	No. of cases n	(%)	Median (range)	Objective survival (months)	p-value (< 0.05)
PC-PI	122		2.6 (0.4–4.8)		0.031
< 2.9	75	(62%)	2.3 (0.4–2.8)	?	
≥ 2.9	46	(38%)	3.2 (2.9–4.8)	17	
PC-AI	94		5.0 (1.4–24.5)		
≤ 4.4	40	(43%)	3.1 (1.4–4.3)	23	0.022
> 4.4	54	(57%)	6.6 (4.5–24.5)	?	
PC-PI/AI	94		0.5 (0.05–2.6)		
< 0.71	64	(68%)	0.4 (0.05–0.70)	?	0.032
≥ 0.71	30	(32%)	1.2 (0.71–2.60)	16	

PC-PI – plasma cell propidium iodide index, PC-AI – plasma cell annexin-V index, PC-PI/AI – plasma cell propidium iodide/annexin-V ratio, ? – still survive at the time of analysis.

more favourable prognosis, at the time of analysis with still unidentifiable OS median (Tab. 1, Fig. 3). The calculated discrimination values of PC-PI, PC-AI and PC-PI/AI stratified a cohort under study into separate asymmetric groups, where the group with unfavourable prognosis (bad risk) and a short overall survival always represented minority (PC-PI 38%, PC-AI 43% and overall PC-PI/AI 32%).

Discussion

MM is characterized by the accumulation of malignant plasma cells in the bone marrow caused primary by failure of normal homeostatic mechanisms to prevent the expansion of postgerminal plasma cell [2]. Cell-cycle control, but mainly dysregulation of apoptosis represents major determinants for disrupted plasma cell homeostasis in MM pathogenesis [18]. Accumulation of non-cycling myeloma cells in the bone marrow originates mainly from dysregulation of apoptosis although the expansion of monoclonal myeloma cells cannot be achieved without dysregulation of the cell cycle earlier during MM initiation. MM is really an accumulative rather than a true proliferative disease [19, 20] and MM constitutes perhaps one group of cells, which cannot undergo apoptosis in the bone marrow [9]. With regard to the above mentioned reasons, an analysis of these processes is crucial for understanding of biological nature of MM as well as for the evaluation of clinical stages of the disease. In MM the overall survival and/or the duration of the response to therapy varies substantially and is significantly influenced by kinetic properties i.e. proliferative and mainly apoptotic characteristics of the plasma cell clone [4–6, 8, 12, 18, 21, 22]. It is well known, that patiens with a high percentage S-phase, i.e. high plasma cell labeling index (PC-LI) have a very bad prognosis with short OS, while those with a low PC-LI usually have a substantially longer OS [3, 21, 23]. PC-LI is an independent and one of the most important prognostic factors and specific markers of MM aggressivenes [4, 6, 8, 18, 23] and a marker for early disease progression and death [4, 24, 25]. Since PC-LI value refers to the whole myelomatous population, but not to growth fraction (GF) alone, even a very small variation (e.g. 1%) denotes substantial changes in GF proliferative activity [26]. The proliferation of human myeloma cells is mainly within a tiny compartment of plasmablastic [18] or primitive plasma cells, that

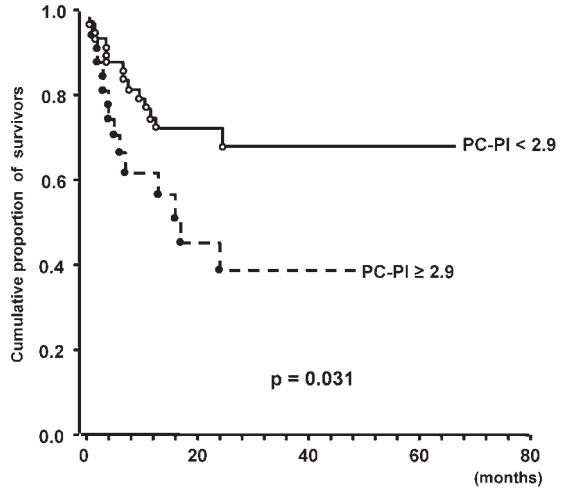

Figure 1. Curves of overall survival according to Kaplan-Meier in the group of 122 patients divided according to propidium iodide proliferation index with cut off value of PC-PI at 2.9% (p=0.031).

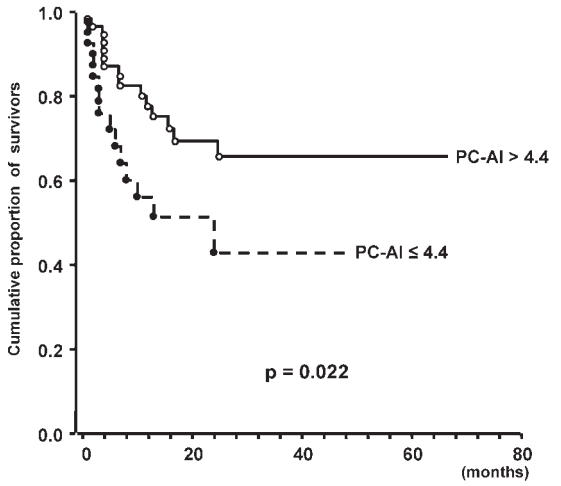

Figure 2. Curves of overall survival according to Kaplan-Meier in the group of 94 patients divided according to annexin-V index with cut off value of PC-AI at 4.4% (p=0.022).

have a higher labelling index than mature (CD_{38++}, CD_{45++}) cells, while primitive plasma cells best correlate with disease activity [5, 27]. In MM, the neoplastic population consists of 3 clonally related compartments [26]: small self-maintaining stem-cell compartment, growth fraction compartment and the largest differentiation compartment where proliferation stops and differentiation into mature plasma cells takes place, and cell loss by apoptosis and necrosis occurs in this compartment. The balance between GF and differentiation compartments determines clinical phases of the disease (progression/relapse, plateau phase and phase of response to therapy). The proliferative activity of the malignant plasma cells is assessed either by PC-LI with bromodeoxyuridine immunofluorescent method or by flow cytometry with propidium iodide (PC-PI) [3, 15, 23, 25]. Propidium iodide staining and flow cytometric analysis used in the present study permits the identification of cellular distribution along the different cell cycle phases. PC-PI analysis is based on the assumption that S-phase cells have a DNA content that is between that of the G_0/G_1-phase and the G_2/M-phase populations [15, 28]. In our previous study we confirmed, in agreement with several reports [5, 9, 29] a high importance of PC-proliferation index examination for differentiation of active (progressive/relapse) versus stable/plateau forms of MM or MGUS and smoldering MM respectivelly [17, 30]. The present study dealing with the actual stage of proliferation and apoptosis of myeloma plasma cells proved clearly the relation between proliferation activity of these cells and prognosis of the disease. Comparison with the results of previous analyses using autoradiographic method with H_3thymidine and later also immunofluorescence method with bromodeoxyuridine confirmed that the flow cytometry method using propidium iodide is technically suitable, quick and reliable [6, 17, 27].

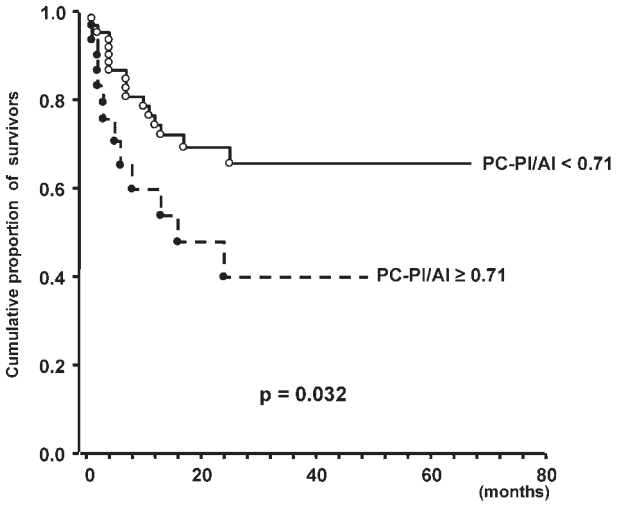

Figure 3. Curves of overall survival according to Kaplan-Meier in the group of 94 patients divided according to overall kinetic index of plasma cells, i.e. PC-PI/AI ratio with cut off value at 0.71 (p=0.032).

The results obtained from a sequence prognostic analysis indicated that the optimal discrimination value of PC-PI is 2.9%, which is very close to that of 3% recommended in pilot studies [6, 15]. The described cut off allows the division of patients into prognostically different groups (62% good and 38% poor risk) with a high statistical significance. BOCCADORO observed improved OS in patients with high PC-LI treated using HD-therapy (HDT) with autologous stem cell transplantation (ASCT) support than in patients receiving conventional therapy. Hence the improved survival abolishes the difference from patients with low PC-LI, he believes this proliferation index to be no longer an adverse prognostic factor in patients treated with HDT with ASCT [21]. This hypothesis should be verified. It is known that proliferation rate of myeloma cells is also associated with bone marrow angiogenesis and tumor burden, with other markers of plasmocyte proliferation activity, e.g. serum thymidine kinase level, with presence of abnormal clone in cytogenetic analysis [31] and with another standard MM prognostic factors, e.g. anemia, hypercalcemia, β_2-microglobulin and creatinine serum level and number of peripheral blood natural killer cells [6, 17, 20, 30, 32].

The expansion of a neoplastic clone is determined by the equilibrium between proliferation on the one hand, and induction or blockade of apoptosis on the other. This blockade causes failure of apoptosis with a prolonged life span of myeloma cells, which could contribute to tumor burden without increasing cell proliferation [3, 26]. In MM, cell death avoidance is likely a more important factor, since myeloma is characterized by a latent accumulation of "resting" plasma cells, which are not in cycle and typically display a very low proliferative activity. The data point to a loss of apoptotic control in MM cells as a consequence of dysregulation of multiple survival pathways stemming from oncogenic transformation and interaction with the bone microenvironment. Numerous combinations of antiapoptotic mechanisms of both intrinsic and extrinsic origin are operating in MM, and their individual contribution may vary in different phases of the disease to ensure the survival of a malignant clone. Control in the apoptotic cascade involves a permeability transition of the mitochondria and the release of cytochrome-c into the cytoplasmic space to form the activation of down-stream effectors [2, 33]. Overexpression of bcl-2 preventing the terminal steps of the apoptotic cascade efficiently inhibits apoptosis by cytokine deprivation (e.g. TNF-α, Fas/Fas ligand, i.e. CD_{95}/CD_{95L}) as well as the cytotoxicity of chemotherapeutic drugs [7, 34, 35]. In myeloma cell apoptosis, many factors are involved, e.g. induction of Fas, Trail (TNF-related apoptosis inducing ligand), the IL-6/IL6R signaling pathway, enhanced expression of Mcl-1 (myeloid cell factor-1), Bcl-XL, INF-κB (nuclear factor kappa B), overexpression of cyclin D and inactivation of cyclin-dependent kinase inhibitor $p16^{INK4a}$, FGFR3 (fibroblast growth factor receptor 3), IGFs (insulin-like growth factor) and many other mediators [1, 36–39].

Similarly to our previous studies, the rate of apoptosis of myeloma plasma cells was evaluated by means of a staining technique with annexin-V FITC, because propidium iodide provides a rapid and simple method of detecting early apoptotic processes [40]. On the basis of sequence prognostic analysis, the most suitable cut off for prognosis assessment of MM patients was determined at the value of PC-AI 4.4%. This value allows division of patients into two considerably different prognostic groups, i.e. good risk – with still unidentifiable OS median, and poor risk – with OS median of only 23 months. We consider the evidence of a significant prognostic importance of the determined index of plasma cell apoptosis at MM diagnosis provided in the present study to be important with regard to the absence of a similar finding in available literature. It is interesting that the statistical significance was even higher than in the case of plasma cell proliferation index. This finding indicates that under conditions of conventional chemotherapy, internal biological nature of plasma cells and microenvironmental factors of bone marrow of a given individual play a decisive role in the disease stage and prognosis. This finding stimulates further investigation of other MM aspects, e.g. achievement and duration of therapeutic response, evaluation of relation to standard prognostic MM factors and verification of prognostic importance of PC-AI even under conditions of HDT supported by ASCT.

Several previous studies reported an inverse relationship between bcl-2 expression and the proliferation of plasma cells. They described that bcl-2 positive cells proliferated minimally or not at all [18, 37]. These observations support the hypothesis that all cycle kinetics may be intimately related to the regulation of programmed cell death, although the nature of this association is not well defined [1, 35]. Myeloma cell immortalization may be, firstly, a consequence of unending proliferation or, secondly, due to prolonged survival through a failure of cell death or apoptosis [34]. This hypothesis as well as the existence of mutual inverse relationship between proliferation and apoptosis described in our previous study [17] explain to a certain extent why the overall kinetic index of plasma cells calculated from proliferation and apoptotic index (PC-PI/AI) ratio did not result in better prognostic prediction compared to the results obtained from separate evaluation of both indices. It was found that within the division of a cohort into two prognostically different groups (good a bad risk), OS median of the bad risk group, characteristics of survival curves as well as statistic relationship were in overall PC-PI/AI similar to those of PC-PI and PC-AI alone.

Conclusion

Our results indicate that not only proliferative but also apoptotic disturbance is substantially important in MM clinical manifestation and course and namely in prognosis of this disease. The results of this study support our previously published working hypothesis of "inverse relation between the proliferation (PC-PI) or apoptotic (PC-AI) activities in plasma cell compartments of patients with MGUS and various forms of MM" [17]. This hypothetic concept has not only potentially pathogenetic, but mainly clinical and therapeutic implication.

References

[1] LANDOWSKI TH, DALTON WS. Molecular biology of plasma cell disorders. In: Mehta J, Singhal S, editors. Myeloma. London: Martin Dunitz, 2002: 25–37.

[2] ZHANG B, GOJO I, FENTON RG. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. Blood 2002; 99: 1885–1893.

[3] GREIPP PR, LUST JA, O'FALLON WM, KATZMANN JA, WITZIG TE, KYLE RA. Plasma cell labeling index and beta$_2$-microglobulin predict survival independent of thymidine kinase and C-reactive protein in multiple myeloma. Blood 1993; 81: 3382–3387.

[4] BOCCADORO M, MARMONT F, TRIBALTO M, FOSSATI G, REDOGLIA V et al. Early responder myeloma: kinetic studies identify a patient subgroup characterized by very poor prognosis. J Clin Oncol 1989; 7: 119–125.

[5] JOSHUA D, PETERSEN A, BROWN R, POPE B, SNOWDON L, GIBSON J. The labelling index of primitive plasma cells determines the clinical behaviour of patients with myelomatosis. Brit J Haematol 1996; 64: 76–81.

[6] SAN MIGUEL JF, GARCÍA-SANZ R, GONZÁLEZ M, MORO MJ, HERNÁNDEZ JM et al. A new staging system for multiple myeloma based on the number of S-phase plasma cells. Blood 1995; 85: 448–455.

[7] SCUDLA V, BACOVSKY J, VYTRASOVA M, ORDELTOVA M, PAPAJIK T, OPICHALOVA D. Prognostic factors and clinical staging systems in multiple myeloma in the group of 237 patients from the years 1991-2002 treated with conventional therapy I. Prognostic significance of selected clinical and laboratory markers. II. Prognostic significance of some existing staging systems. Klin Onkol (Brno) 2002; 15 Suppl 1: 7–14, 15–20.

[8] TRENDLE MC, LEONG T, KYLE RA, KATZMANN JA, OKEN MM et al. Prognostic significance of the S-phase fraction of light-chain-restricted cytoplasmic immunoglobulin (clg) positive plasma cells in patients with newly diagnosed multiple myeloma enrolled on Eastern Cooperative Oncology Group treatment trial E9486. Am J Hematol 1999; 61: 232–237.

[9] WITZIG TE, TIMM M, LARSON D, THERNEAU T, GREIPP PR. Measurement of apoptosis and proliferation of bone marrow plasma cells in patients with plasma cell proliferative disorders. Brit J Haematol 1999; 104: 131–137.

[10] XU JL, LAI R, KINOSHITA T, NAKASHIMA N, NAGASAKA T. Proliferation apoptosis, and intratumoral vascularity in multiple myeloma: correlation with the clinical stage and cytological grade. J Clin Pathol 2002; 55: 530–534.

[11] SWOG Southwest Oncology Group. Remission maintenance therapy for multiple myeloma. Arch Int Med 1975; 135: 147–152.

[12] DURIE BGM, SALMON SE. A clinical staging system for multiple myeloma. Cancer 1975; 36: 842–854.

[13] WIJDENES J, VOOIJNS WC, CLÉMENT C, POST J, MORARD F et al. A plasmocyte selective monoclonal antibody ($B\text{-}B_4$) recognize syndecan-1. Brit J Haematol 1996; 94: 318–323.

[14] ORDELTOVA M, SPIDLOVA A, SCUDLA V. Cell-cycle S-phase evaluation (CD_{138}/propidium iodide index) of myeloma cells using flow cytometry. Diagnoza 1999; 1: 12–15.

[15] ORFAO O, GARCIA-SANZ R, LÓPEZ-BERGES MC, VIDRIALES MB, GONZÁLEZ M et al. A new method for the analysis of plasma cell DNA content in multiple myeloma samples using a CD_{38}/propidium iodide double staining technique. Cytometry 1994; 17: 332–339.

[16] SCUDLA V, ORDELTOVA M, SPIDLOVA A, BACOVSKY J, KURASOVA J, VRANOVA V. Importance of examination of the propidium-iodide index of plasmocytes in multiple myeloma. I. Relationship to some laboratory findings of the disease. II. Relationship with extent and activity of the disease. Vnitr Lek 1999; 45: 331–335, 336–341.

[17] SCUDLA V, ORDELTOVA M, BACOVSKY J, VYTRASOVA M, SUMNA E et al. A contribution to examination of propidium iodide and annexin V plasma cells indices in multiple myeloma. Neoplasma 2003; 50: 363–371.

[18] CHEN Q, GONG B, MAHMOUD-AHMED AS, ZHOU A, HSI ED et al. Apo_2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. Blood 2001; 98: 2183–2192.

[19] BATAILLE R. A cellular model for myeloma cell growth and differentiation. Hematol J 2002; 3 Suppl 2: 48.

[20] SCHAMBECK CM, BARTL R, HÖCHTLEN-VOLLMAR W, WICK M, LAMERZ R, FATEH-MOGHADAM A. Characterization of myeloma cells by means of labeling index, bone marrow histology, and serum β_2-microglobulin. Hematopathology 1996; 106: 64–68.

[21] BOCCADORO M, TARELLA C, PALUMBO A, ARGENTINO CH, TRIOLO S et al. An analysis of which subgroups of multiple myeloma patients, divided according to β_2-microglobulin and plasma cell labeling index, benefit from high dose vs conventional chemotherapy. Haematologica 1999; 84: 905–910.

[22] CALIGARIS-CAPPIO F. Biology of the malignant plasma cell. In: Gahrton G, Durie BGM, Samson DM, editors. Multiple myeloma and related disorders. London: Arnold, 2004: 58–73.

[23] GREIPP PR, WITZIG TE, GONCHOROFF NJ, HABERMANN TM, KATZMANN JA, O'FALLON WM. Immunofluorescence labeling indices in myeloma and related monoclonal gammopathies. Mayo Clin Proc 1987; 62: 969–977.

[24] STEENSMA DP, GERTZ MA, GREIPP PR, KYLE RA, LACY MQ, LUST JA et al. A high bone marrow plasma cell labeling index in stable plateau-phase multiple myeloma is a marker for early disease progression and death. Blood 2001; 97: 2522–2523.

[25] LOKHORST HM, BOOM SE, BAST BE. Determination of the plasma cell labelling index with bromodeoxyuridine in a double fluorescence technique. Brit J Haematol 1986; 64: 271–275.

[26] VACCA A, RIBATTI D, RONCALI L, DAMMACCO F. Angiogenesis in B cell lymphoproliferative diseases. Biological and clinical studies. Leuk Lymph 1995; 20: 27–38.

[27] DREWINKO B, ALEXANIAN R, BOYER H, BARLOGIE B, RUBINOW SI. The growth fraction of human myeloma cells. Blood 1981; 57: 333–338.

[28] GARCIA-SANZ R, ORFAO A, GONZALEZ M, MORO JM, HERNÁNDEZ JM et al. Prognostic implications of DNA aneuploidy in 156 untreated multiple myeloma patients. Br J Haematol 1995; 90: 106–112.

[29] JOSHUA DE, SNOWDON L, GIBSON J, ILAND H, BROWN R et al. Multiple myeloma: plateau phase revisited. Haematol Rev Comm 1991; 5: 59–66.

[30] GREIPP PR, KYLE RA. Clinical, morphological, and cell kinetic differences among multiple myeloma, monoclonal gammopathy of undetermined significance, and smoldering multiple myeloma. Blood 1983; 62: 166–171.

[31] RAJKUMAR SV, FONSECA R, DEWALD GW, THERNEAU TM, LACY MQ et al. Cytogenetic abnormalitis correlate with the plasma cell labeling index and extent for bone marrow involvement in myeloma. Cancer Genet Cytogenet 1999; 113: 73–77.

[32] KYLE RA. Multiple myeloma. Diagnostic challenges and standard therapy. Sem Hematol 2001; 38 Suppl 3: 11–14.

[33] KLUCK RM, BOSSY-WETZEL E, GREEN DR, NEWMEYER DD. The release of cytochrome-c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997; 275: 1132–1136.

[34] LANDOWSKI TH, QU N, BUYUKSAL I, PAINTER JS, DALTON WS. Mutations in the Fas antigen in patients with multiple myeloma. Blood 1997; 90: 4266–4270.

[35] STECK K, MCDONNELL T, SNEIGE N, EL-NAGGAR A. Flow cytometric analysis of apoptosis and BCL-2 in primary breast carcinomas: clinical and biological implications. Cytometry 1996; 24: 116–122.

[36] EGLE A, VILLUNGER A, MARSCHITZ I, KOS M, HITTMAIR A et al. Expression of Apo-1/Fas (CD_{95}), Bcl-2, Bax and Bcl-x in myeloma cell lines: relationship between responsiveness to anti-Fas mab and p53 functional status. Brit J Haematol 1997; 97: 418–428.

[37] PUTHIER D, PELLAT-DECEUNYNCK C, BARILLE S, ROBILLARD N, RAPP MJ et al. Differential expression of Bcl-2 in human plasma cell disorders according to proliferation status and malignancy. Leukemia 1999; 13: 289–294.

[38] YANG J, LIU X, BHALLA K, KIM CN, IBRADO AM et al. Prevention of apoptosis by BCL-2: release of cytochrome-c from mitochondria blocked. Science 1997; 275: 1129–1132.

[39] LANDOWSKI T, GLEASON-GUZMAN M, DALTON W. Selection for drug resistance to Fas-mediated apoptosis. Blood 1997; 89: 1854–1861.

[40] KOOPMAN G, REUTELINGSPERGER CPM, KUIJTEN GAM, KEEHNEN RMJ, PALS ST, VAN OERS MHJ. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994; 84: 1415–1420.