Application of flow cytometry immunophenotyping and multidrug resistance assay in B-cell acute lymphoid leukemia and multiple myeloma*

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Multidrug resistance is one of the mechanisms how to explain failure of chemotherapy in patients with different hematological malignancies. In this study we aimed to evaluate and compare the drug resistance in B-cell acute lymphoid leukemia (B-ALL) and multiple myeloma (MM) in association with their immunophenotypes and genotypes. Eleven patients with B-ALL and 14 patients with MM were classified according to prognostic factors. Standard MoAb panel for ALL and triple labeled antibodies (CD38/CD56/CD19) and detection of intracellular light chains for MM were used. Flow cytometric calcein assay was performed for measure of P-glycoprotein (MDR-1) and multidrug resistance associated protein (MRP-1) activity. Markers CD19, CD20 and HLA-DR proved to be useful in identifying cells of B-lymphoid lineage. CD34 progenitor cell antigen was present in high proportion of ALL blasts. Both the abnormal plasmacell populations and their monoclonality in MM were confirmed by immunophenotyping, too. The mean MDR activity factor (MAF) values were not different in patients with MM and B-ALL. However, the mean MRP-1 values in MM were significantly lower than MAF-MDR-1 (1.85 ± 3.8 versus 5.92 ± 7.45, $p=0.05$), but we have found lower values in refractory conditions as expected from previous studies of acute myeloid leukemia. The immunophenotyping was helpful in detection of abnormal populations showing no correlation with the MDR. However, in this study we could not confirm high MDR activity despite of the failure of chemotherapy. The calcein assay seems to be useful for quantitative and sensitive measurement of the MDR proteins. The low activity of MDR-1 and MRP-1 in MM need further clarification, indicating the involvement of different transport in the resistance mechanism.

Key words: multiple myeloma, acute lymphoid leukemia, immunophenotype, intracellular light chain, multidrug resistance

Classification of B-cell malignant lymphomas is based on the recent World Health Organization (WHO) adaptation of the Revised European-American Lymphoma Study Group [10]. B-cell malignancies include both acute leukemia (precursor B-cell neoplasm), more mature B-cell lymphomas, as well as plasma cell myeloma (mature or peripheral B-cell neoplasm). The B-lineage neoplasms are characterised by different cell surface and/or intracytoplasmic markers. However, most cells acquire also genetic abnormalities that are specific to each lymphoma subtype [27].

Acute lymphoblastic leukemias (ALL) are the most common malignancies of childhood. They represent one of the more successful areas for chemotherapy with cure rate now approaching 75% [11, 19]. In contrast, in adult ALL complete remission rates of 80–85% and leukemia-free survival rates of 30–40% can be achieved [11]. Of the lymphoblastic leukemias/lymphomas, only 20% are of T-cell origin. However, more than 80% of mature lymphoid neoplasias (mature cell non-Hodgkin lymphomas) arise from B-cell lineage. Consideration of multiple myeloma falls naturally after B-cell non-Hodgkin's lymphomas, from which they are not sharply demarcated [25, 27].

The immunological subtypes of ALL show considerable differences in terms of presentations, clinical course and relapse risk. They can be subdivided into standard and

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high-risk groups with significantly different outcomes. Adverse prognostic factors in adult B-ALL include higher age (>50 yrs), high WBC (>300.00/μl), pro-B immunophenotype (B-lineage+, CD10-), cytogenetic/molecular genetic abnormality (t(9;22)BCR-ABL, t(4;11)/ALL1-AF4), late achievement of complete remission and minimal residual disease (MRD) [11, 21].

Multiple myeloma (MM) belongs to peripheral B cell malignant lymphomas, too. MM is a severely debilitating, incurable, and uniformly fatal neoplastic disease of B-cell origin. Its prognosis has been relatively unchanged over the past 30 years, except for a subgroup of patients undergoing successful autologous or allogeneic stem cell transplantation. However, studies over the past 15 years provided important findings regarding the role of osteoclast activating factor, interleukin-6, interleukin-1β, tumour necrosis factor-α, Bcl-2 family proteins and vascular endothelial growth factor which augment growth, trigger migration, stimulate angiogenesis and protect against drug induced apoptosis in MM [1].

Multidrug resistance is recognised clinically as the development of tumour resistance to a wide variety of antineoplastic drugs. Anticancer treatments such as drugs, xenobiotics, or radiation must hit their cellular targets and then cause some form of cellular alteration or damage. Rather then killing directly, it is thought that in most cases the damage inflicted by the anticancer agent triggers the process of programmed cell death, apoptosis. Resistance to multiple drugs may arise from cellular defence that broadly limit the access of the agents to their cellular targets (for example drug transporters), or that prevent the cells from entering apoptosis, following injury.

Recent molecular research in multidrug resistance (MDR) resulted in the isolation and characterisation of genes coding for several proteins associated with MDR, including P-glycoprotein (P-gp), multidrug resistance associated protein (MRP), and more recently the breast cancer resistance protein (BCRP/MXR) [22]. These proteins are expressed at varying degrees in different hematological neoplasms and P-gp proved to be an independent prognostic factor, predicting relapse in childhood acute leukemia [6, 23].

In our previous studies the diagnostics of multidrug resistance in cancer [26] and the usefulness of calcein assay for detection of multidrug resistance in acute myeloid leukemia were described [16]. In this study we aim to evaluate the drug resistance in B-cell acute lymphoid leukemias and multiple myeloma.

Patients and methods

Patients. We collected bone marrow samples from 11 patients with B-cell acute lymphoid leukemia (B-ALL), (6 females, 5 males, mean age at the diagnosis 35.9 ± 15.4 years) and 14 patients with multiple myeloma (MM) (11 females, 3 males, mean age 62.0 ± 14.0 years) diagnosed at the National Medical Centre and St. Rokus Hospital, Budapest. Clinical data of patients were grouped according to the diagnosis (ALL versus MM) (Tab. 1 and 2).

Table 1. Characteristics of patients with B-ALL

No.	Sex	Age years	WBC x 10^9/l	LDH U/l	Cytogenetics (at diagnosis)
1	M	44	100.0	1785	Ph+
2	M	18	22.0	755	NA
3	F	16	35.0	7010	Komplex abnormality
4	F	38	44.0	1188	Normal
5	F	55	4.7	484	Normal
6	M	22	20.0	555	Normal
7	F	16	1.2	273	Hyperdiploid
8	F	39	47.0	937	Ph+
9	F	58	10.7	625	Normal
10	M	45	28.5	2020	ND
11	F	44	5.8	500	Normal

M – male, F – female, WBC – white blood cell count, LDH – lactate dehydrogenase, Ph+ – Philadelphia chromosome positive, NA/ND – not available/not done, ALL – acute lymphoid leukemia.

The MM patients were classified according to the Durie-Salmon and WHO criteria [7, 10]. Prognostic factors as bone marrow plasma cell infiltration, level of the immunoglobulin subtype, β2-microglobulin, lactate dehydrogenase, white blood cell count were determined by standard laboratory procedures.

Immunophenotyping. Bone marrow cells were analyzed by flow cytometry (Becton-Dickinson FacsCalibur) using two- and three-colour immunofluorescence labeling (FITC/PE/PerCP). Lyse-wash preparation method was used with a panel of antibodies (CD45/CD14, CD7/CD3, CD10/CD20, CD19/HLA-DR, CD34/HLA-DR, CD38/IC-kappa, CD38/IC-lambda, CD38/CD56/CD19 (Becton Dickinson, San Jose, CA; Dako, Glostrup, DK; Caltag Laboratories Burlingame, CA). Surface antigen expressions were analyzed with additional one-step intracellular light chain (LC) detection by CHUNG et al [3].

Cytogenetic analysis. Cytogenetic studies on bone marrow were performed using standard G-banding with trypsin-Giemsa or trypsin-Wright's staining. ICSN 1995 guidelines were followed for clonal definition and description of individual and numerical karyotypic anomalies. Twenty metaphases were analyzed [15].

MDR-1 (P-glycoprotein) and MRP-1 (multidrug resistance-associated protein) function analysis. For functional assay of MDR1 and MRP-1 activity the flow cytometry calcein assay was used as described previously [14]. Briefly, lymphocytes and leukemic blasts were isolated by Ficoll gradient (Histopaque-1077, Sigma, St Louis, MO) and washed twice in phosphate buffered saline (PBS). 5x10^5

Table 2. Characteristics of patients with MM

No.	Sex	Age years	Stage	IFE	Cytogenetic	BM-PC %	Previous therapy	Disease state
1	M	47	I/A	non-secr.	normal	15	VAD+ASCT	Rel.
2	M	38	II/A	IgG-λ	ND	15	ICOMP+ASCT	Rel.
3	F	44	II/A	IgA-λ	normal	20	VAD	PR
4	F	65	I/A	IgG-λ	ND	6	ND	Stable
5	M	52	II/A	IgA-κ	ND	30	ICOMP,VAD	Refr.
6	F	63	II/B	IgG-κ	NA	20	ICOMP,VAD	PR
7	F	49	II/A	IgG-κ	normal	30	ICOMP,IFN,VAD	Rel.
8	F	76	III/A	IgA-λ	12-, +2	80	MP	Refr.
9	F	76	III/B	IgA-λ	NA	30	MP	Refr.
10	F	65	II/A	IgG-κ	normal	80	ICOMP	Rel.
11	F	78	II/A	IgG-λ	normal	60	MP	Refr.
12	F	63	II/A	IgA-κ	normal	30	ND	De novo
13	F	83	III/A	IgG-κ	normal	80	MP	Refr.
14	F	70	II/A	IgG-κ	13q-,8p-	40	MP	Rel.

M – male, F – female, IFE – immunofixation electrophoresis, BM-PC –bone marrow-plasma cell percent, ND/NA – not done/not available, VAD – vincristin, doxorubicin, dexamethasone, ASCT – autologous stem cell transplantation, M – melphalan, P – prednisone, ICOMP – idarubicin, cyclophosphamid, vincristin, melphalan, prednisone, IFN – interferon, Rel – relapsed, PR – partial remission, Refr. – refractory.

Table 3. Results of the multiparametric flow cytometry in patients with B-ALL

No.	CD19+ %	CD20+ %	CD10+ %	CD34+ %	HLA-DR+ %	MAF-MDR1 %	Follow-up months
1	ND	ND	ND	+++	ND	0	15
2	++	+	–	–	+	7	35
3	–	++	–	+	+	0	30
4	++	+	–	–	++	0	1
5	+	+	–	+	+++	15	18
6	–	+	–	+++	+	14	74
7	+++	–	+++	+++	+++	0	75
8	–	+++	–	+++	+++	13	69
9	+++	++	–	+++	+++	1	60
10	+++	+++	–	+++	+++	18	14
11	+++	++	+++	+++	+++	0	34

Value of the expression: – <25%, + 25–50%, ++ 50–75%, +++ >75%.

cells were preincubated for 5 minutes at room temperature in HPMI medium (pH 7.4) containing 60 μM verapamil or its solvent HPMI. Simultaneously, 10 μM MK-571as MRP-1 inhibitor was used selectively. The cells were then incubated with 0.25 μM calcein-AM (Molecular Probes, Eugene, OR) for 10 min at 37 ˚C. Following a fast centrifugation (15 sec, 14 000 rpm) cells were resuspended and 5 μg/ml propidium-iodide (PI) was added to investigate cell viability. The calcein accumulation was measured on FacsCalibur flow cytometer (Becton Dickinson Biosciences. San Jose, CA). The data were analyzed by using the mean fluorescence intensity (MFI) values of the selected cells (blast in ALL and strong CD38+ in MM) in the presence and absence of an inhibitor (verapamil and MK-571). The quantitative measure of transport activity, the MDR activity factor (MAF) was determined by the formula: MAF= 100 x (MFI_v-MFI_o)/ MFI_v as previously described [12, 13]. There was investigated only activity of MDR-1 protein in ALL, because some study has recorded that role of MRP-1 is less than MDR1 in acute leukemias [4, 17].

Results

The results of immunophenotyping and MAF of ALL group are shown in Table 3. Markers CD19, CD20 and HLA-DR proved to be useful, in association with morphological and histochemical analysis in identifying cells of B-lymphoid lineage. CD34 progenitor cell antigen was present in high proportion of ALL blasts in seven cases, however in two cases the CD34 expression was low and in additional two cases it was negative. The pan-B-cell antigen CD20 was found to be co-expressed with HLA-DR in seven out of ten samples. High expression of CD10 antigen was detected in

Table 4. Results of the flow cytometry and MAF in patients with MM

No.	IC-LCκ+/ CD38^{bright}+ %	IC-LCλ+/ CD38^{bright}+ %	CD38+/ CD56+/ CD19-	CD38+/ CD56+/ CD19+	MAF- MDR1+ %	MAF- MRP1+ %
1	75	25	+++	negative	0	0
2	29	71	negative	+++	25	0
3	14	86	+++	negative	14	0
4	6	94	+++	negative	5	0
5	94	6	+++	negative	6	0
6	95	5	+++	negative	0	0
7	26	74	++	negative	0	0
8	7	93	+++	negative	12	11
9	6	94	+++	negative	12	10
10	82	18	+++	negative	0	0
11	20	80	+++	negative	0	0
12	98	2	++	negative	3	5
13	91	9	+++	negative	0	0
14	95	5	ND	ND	6	0

IC-LC – intracytoplasmic light chain, IC-LCλ+/CD38^{bright}+ – IC-LCλ positive cells within the CD38^{bright} positive population, IC-LCκ+/CD38^{bright}+ – IC-LCκ positive cells within the CD38^{bright} positive population, ND – not done; – <25%, + 25–50%, ++ 50–75%, +++ >75%, MDR1 – P-glycoprotein (P-gp), MRP1 – multidrug resistance associated protein 1

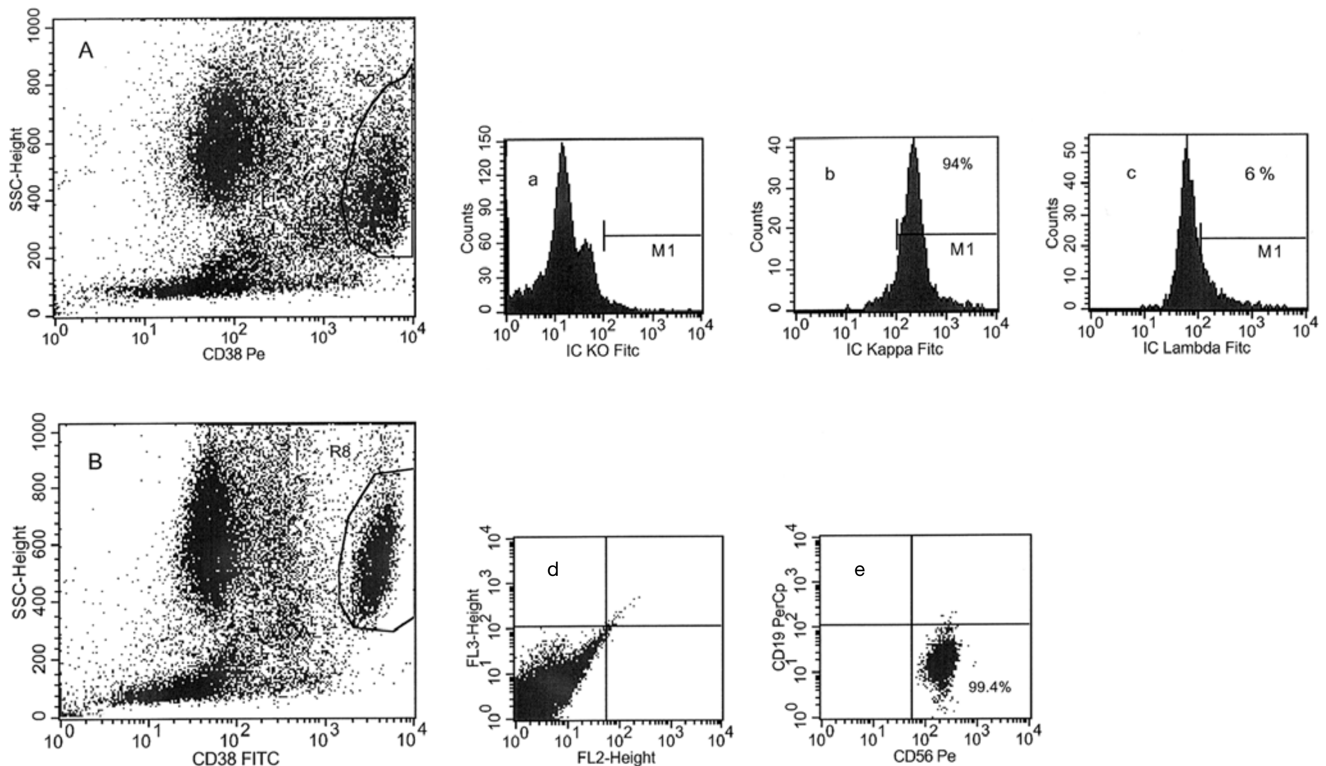

Figure 1. Immunophenotyping of patient No. 5 with multiple myeloma. Upper left (A) and lower left picture (B) show bright CD38+ cells. The upper right histograms (b, c) show expression of intracytoplasmic light chains with auto-control (a). The kappa expression was increased (b). The lower right pictures (dot-plot) show expression of CD56+/CD19- (e) with control (d) on the bright CD38+ cells.

two cases. The MAF values including the two Ph+ cases were not different from that found in normal mononuclear cells (n=40, mean $\pm$ SE = 11.9 $\pm$ 6.4) confirming the previous results reported by KARASZI et al [16].

Monoclonality in the serum was determined by immunofixation (IFE) (Tab. 2) and by immunophenotyping in the BM cells (Tab. 4) detecting co-expression of CD38 and intracytoplasmic light chain molecules. The IFE negative

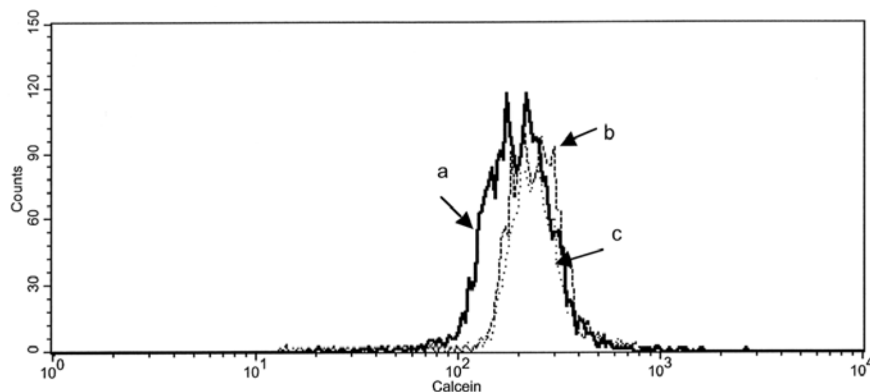

Figure 2. Calcein accumulation of patient No. 12 without and with inhibitors; a – without inhibitor, b – with verapamil, c – with MK-571.

non-secretory MM case (No. 1) proved to be kappa positive by immunophenotyping. One case (No. 7) showed kappa light chain by IFE and lambda positivity by immunophenotyping. In the rest of cases the serological monoclonality was confirmed by immunophenotyping, too. The CD38 and CD56 double positive cells were regarded as abnormal plasma cells (Fig. 1). The abnormal plasma cell population was negative on CD19 antigen, but one case (No 2) showed triple positive (CD38/CD19/CD56) cell surface antigen pattern. There was no correlation among the monoclonal light chain expression, the percentage of CD56/CD38 antigen double positive plasma cells and the MAF values determined both by MDR1 and MRP1 methods. Chromosomal aberrations were detected in two cases with clinical significance (1 relapse, 1 refractory).

The mean MAF-MDR1 (P-gp) values were not different in patients with multiple myeloma and B-ALL (mean $\pm$ SD = 5.92 ± 7.45 and 6.18 ± 7.37, respectively). However, the mean MAF-MRP1 values in MM were significantly lower than MAF-MDR1 (1.85 ± 3.8 versus 5.92 ± 7.45, p=0,05), but both transport activities were lower than it was expected by calculating the refractory status (Fig. 2). We have not found any correlation between MAF-MDR1, MRP1 values and other clinical prognostic factors of MM (including Ca+, LDH, creatinine and presence of bone disease). Probability of survival was not evaluated because of small number of cases in both groups.

Discussion

Both B-cell ALL and multiple myeloma can be regarded as monoclonal B-cell diseases. Leukemic cells in adult B-ALL are characterized as very immature, predominantly CD10- abnormal B-cells corresponding to the early normal B-cell differentiation. Multiple myeloma, however, is a monoclonal plasma cell disease characterized by the proliferation of plasma cells corresponding to the most mature B-cell differentiation stage synthesizing a monoclonal antibody (M-protein). In MM some authors emphasized the superiority of a sensitive flow cytometry assay as compared to immunofixation for the separation of normal and neoplastic plasma cells [20]. In addition, SAHARA et al found a strong correlation between CD56 expression and the presence of extramedullary disease and demonstrated that overall survival was significantly lower in CD56- than in CD56+ patients [18]. CHANG et al used a two-step acquisition technique to determine the cytoplasmic immunglobulin light chain expression. This method is highly sensitive and able to identify even a minute clonal plasma cell population [3]. Our experience with the immunofixation and cytoplasmic light chain detection within the CD38+ cell population was equally useful for the detection of abnormal light chain produced by plasma cells except one patient (case No. 7). However, in non-secretory MM the immunophenotyping is the only diagnostic method able to detect the abnormal protein (case No. 1). Unlike in pediatric lymphoid leukemia, B-ALL in adults is not a chemotherapy responsive disease and has a poor clinical outcome, likewise MM which is generally regarded as incurable. Therefore, both diseases can be characterized as chemotherapy non-responsive and seem to need drug resistance evaluation.

Despite the heterogeneity of both the B-ALL and MM groups of patients analyzed here, the mean multidrug resistance activity factor (MAF) values were not different from those found in normal mononuclear cells (11.9 ± 6.4) as reported in our previous work [16]. Regarding MM, several studies have shown that MDR is not expressed at diagnosis, but, in contrast, can be observed in patients treated with combined chemotherapy regimen [9]. We were interested in function – not expression – of MDR proteins, because recent reports suggested that it is more important than expression value [28]. However, our results could not confirm these observations showing not significantly elevated, low or barely detectable MAF activity in previously treated and relapsed patients, too (Tab. 4). This observation was made also in the ALL group, analysed individually (Tab. 3). In our MM cases, however, the mean MAF-MRP1 values were significantly lower than MAF-MDR1 (1.85 ± 3.8 versus 5.92 ± 7.45, p=0.05) indicating different transport resistance mechanisms [22]. There was no correlation among the monoclonal light chain expression, and the presence of CD56 antigen and the MAF values determined both the MDR1 and MRP1 in MM.

Numerous *in vitro* and *in vivo* model studies demonstrated that overcome of the resistance improve patient out-

come. Several agents, among others PSC-833, cyclosporin-A, GF120918 and VX-710 are used for this purpose. SONNEVELD et al presented results in the phase III study based on efficacy of cyclosporin-A in patients with refractory multiple myeloma [24]. Not only at diagnosis but, even in refractory disease should be important that clinicians could reach sensitive, quantitative methods for choosing the appropriate treatment for patients.

The flow cytometry assay is able to identify the clonal cell populations and may allow quantitative measurement of the multidrug resistance protein. P-gp, the gene product of MDR1, confers multidrug resistance against anti-neoplastic agents, but it also plays an important role in the bioavailability of commonly used drugs in medical treatment. Various polymorphisms in the MDR1 gene have been identified recently [2, 5, 22]. In addition, MDR1 is only one of several mechanisms through which patients may become refractory to chemotherapy [24].

There are some genetic alterations having functional significance in the generation and progression of MM. In spite of recent advances in this field, the cause and exact molecular genetic basis of MM remain obscure [8].

Based on the results, clinical non-responsiveness in the reported B-ALL and MM cases is not associated with the over-expression of MDR1 or MRP1 therefore other resistance pathway may come into consideration. However, clinical investigations pertaining to the functional role of MDR1 allelic variants for the bioavailability of anti-cancer agents in B-ALL and MM patients would be of great significance.

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