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Differential gene expression of bone marrow CD34+ cells in early and advanced myelodysplastic syndrome

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Myelodysplastic syndrome (MDS) is a hematopoietic stem cell disorder characterized by ineffective hematopoiesis and dysplasia in one or more blood cell lines. Because it often progress to poor outcome stages or acute leukemia we searched for candidate genes associated with disease progression. Using microarrays we performed gene expression profiling in CD34+ cells of 4 early and 4 advanced MDS patients and identified 286 significantly differentially expressed genes between these two categories. Out of these, 136 genes were up-regulated and 150 down-regulated in early MDS compared to advanced MDS. Using clustering analysis those two patient categories were clearly differentiated. Further, we selected three genes (*ADAM8*, *BIRC5*, *MPL*) for gene expression validation by qRT-PCR in an additional set of 29 MDS and sAML patients. We confirmed decreasing trend for *BIRC5* expression from early to advanced stages of MDS, with the lowest levels in sAML patients. On the contrary, higher *ADAM8* and *MPL* expression was observed in most advanced MDS patients compared to the early MDS patients. Association between gene expression levels and bone marrow blast proportion was tested, but only *BIRC5* expression showed negative correlation ($r=-0.83$ at $p<0.001$). This study demonstrates stage-specific expression of some genes that may have potential prognostic significance.

Key words: gene expression profiling, myelodysplastic syndrome, CD34+ cells

Myelodysplastic syndrome (MDS) is a clonal stem-cell disorder characterized by ineffective hematopoiesis, dysplasia of at least one blood cell lineage, peripheral cytopenia and increased potential progress to acute myeloid leukemia (AML) [1, 2, 3]. Etiology of MDS is still unclear; however, several environmental risk factors are known (smoking, benzene, pesticides, toluene, cytotoxic agents or ionizing radiation) [4, 5, 6]. Multistep pathogenesis involves several subsequent processes including development and expansion of mutant clone of a hematopoietic progenitor cell, and transformation into AML in 1/3 of MDS cases.

Two classification systems for MDS have been adopted: the classification according to the French-American-British (FAB) Cooperation Group of 1982 [1] that was recently modified by the World Health Organization (WHO) [7]. Early stages of MDS – refractory anemia (RA), refractory cytopenia with multilineage dysplasia (RCMD) and also the subtypes with the presence of ring sideroblasts (RARS, RCMD-RS) are characterized by better overall survival (~59 months) and lower risk of leukemic development (21%) [8]. In this initial phase, excessive apoptosis in marrow hematopoietic cells leads to ineffective hematopoiesis and peripheral cytopenia in spite of increased proliferation of hematopoietic elements [9]. Advanced MDS subtypes, refractory anemia with excess of blasts (RAEB-1 and 2), are associated with higher probability of leukemic transformation (>50%) and shorter survival of patients (~16 months) [8]. Subtype stratification in MDS is crucial for further treatment management and prognosis. Microarray assays revealed that the MDS subtypes are considerably different at the molecular level and thus gene expression profiling may contribute to diagnosis and/or prognosis specification.

Moreover, MDS represents a useful *in vivo* model of malignant clone development due to frequent leukemic transformation. Comparison of patients with MDS and sAML resulted in definition of several parameters, which correlated with risk of leukemic transformation: age under 40 years, pancytopenia in 3 lineages, >15% bone marrow (BM) blasts, ≥2 abnormal karyotypes and treatment with combined

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chemotherapy [10]. On the molecular basis, several genes were identified as potential prognostic markers. Protein expression of BMI-1 was observed to positively correlate with IPSS score, moreover, early MDS patients with higher percentage of BMI-1+ cells showed disease progression to RAEB [11]. Gene expression of *WT1* is used as a molecular marker in MDS and AML. Its expression correlates with MDS subtype (higher in RAEB and sAML patients), blast percentage and karyotype abnormalities and increases during disease progression [12]. Moreover, *WT1* expression has been introduced as a marker for detection of minimal residual disease [13].

MDS is a disorder arising from the hematopoietic stem cell and thus the interest of most researchers is focused on the hematopoietic progenitors (CD34+, CD133+). Nevertheless, myeloid precursors (CD33+) and non-hematopoietic cells (BM stromal cells, osteoblasts) have been extensively studied. Due to unique expression pattern of each cell population, cell composition of analyzed samples is crucial. We purified CD34+ hematopoietic progenitors from bone marrow of 8 MDS patients and performed gene expression profiling using microarrays. For comparative analysis the patients were grouped into 2 categories (early and advanced MDS) to identify differentially expressed genes between these disease stages.

Patients and methods

Bone marrow (BM) samples (5ml) were collected from 8 MDS patients, each with a diagnosis of primary MDS based on WHO classification system. Patients clinical data are listed in the Table 1. We used a pooled control sample of 2 healthy donors for comparative analysis. The control was labeled and hybridized together with each patient sample onto a particular array slide. The array data were validated by quantitative real-time polymerase chain reaction (qRT-PCR) in an additional set of 29 patients: 5q- syndrome (n=4), RCMD (n=5), RAEB-1 (n=4), RAEB-2 (n=6), AML from documented MDS (n=5), AML with multilineage displasia (AML-MLD) (n=5). For further analysis patients with AML from documented MDS and AML-MLD patients were grouped into one entity named sAML. Informed consent was obtained from all participants.

BM mononuclear cells were isolated by Ficoll-Hipaque density centrifugation (GE Health Care). To minimize differences in cell composition among particular samples, we purified CD34+ hematopoietic progenitors using magnetic separation and specific antibodies (Direct CD34 Progenitor Cell Isolation Kit, MACS; Miltenyi Biotec). Total RNA was extracted by acid guanidin-thiocyanat-phenol-chloroform method [14]. To avoid DNA contamination, samples were incubated with DNase I and afterwards cleaned through the RNeasy MinElute Cleanup Kit columns (Qiagen). Final integrity of total RNA was verified using the Bioanalyzer 2100 instrument and Eukaryote Total RNA Nano Chip (Agilent Technologies). RNA integrity number (RIN) of all samples was 8.3 on average.

Microarray analysis

Gene expression profiling was performed by Human 1A (V2) arrays (Agilent Technologies) covering more than 22 500 transcripts. The Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) was employed to produce fluorescently labeled cRNA following the manufacture's protocol. Cyanine 3-CTP (Perkin-Elmer) was adopted for labeling of the pooled control sample while cyanine 5-CTP (Perkin-Elmer) was incorporated into the patient samples. Unincorporated nucleotides were removed using the RNeasy MiniSpin Columns (Qiagen) and the fluorophore incorporation efficiency was measured by spectrophotometer. Equal amount of fluorescently labeled cRNA from the pooled sample and the patient sample was mixed, fragmented and hybridized onto the oligonucleotide microarray. Hybridization was performed at 60°C for 17 hours at 6 rpm. The microarrays were scanned with GenePix 4000B scanner (Molecular Devices).

The image files were uploaded into ImaGene (BioDiscovery) to convert the images into raw data files. Data from each microarray slide were normalized in R program for statistical computing [15], using package smida [17]. The normalization consisted of intensity-dependent normalization within each microarray slide, followed by quantile normalization between slides. The aim of the experiment was to find differentially expressed genes between two clinical subgroups. For this purpose, a statistical hypothesis testing using T-test between these groups was performed. Next, the empirical Bayesian thresholding was applied [18] to avoid a multiple testing problem.

Table 1. Clinical data of MDS patients. IPSS=International Prognostic Scoring System, BM=bone marrow, WBC=white blood cell count, [n]=number of evaluated mitosis.

Patient	Gender	Age	Diagnosis	Blasts in BM (%)	WBC (10^9/l)	Karyotype	IPSS
1	M	59	RAEB-2	–	2.36	41-44,XY, multiple changes [3]; polyploidy [6]	2
2	M	30	RCMD	–	2.23	46,XY [19]; polyploidy [3]	0.5
3	M	45	RCMD	–	3.55	46, XY [11]	0.5
4	M	57	RAEB-2	16	4.11	44-46,XY, multiple changes [19]	3
5	F	53	RARS	0.8	5.98	46, XX [9]	0
6	M	61	RAEB in progression to AML-MLD	32.8	27.37	46,XY t(8;21) (q22;q22) [4]	-
7	M	65	RAEB 1-2	6.4	3.53	46,XY [12]; 47, XY, +8 [4]	1
8	F	47	RCMD	0.5	1.88	46, XX [8]	0.5

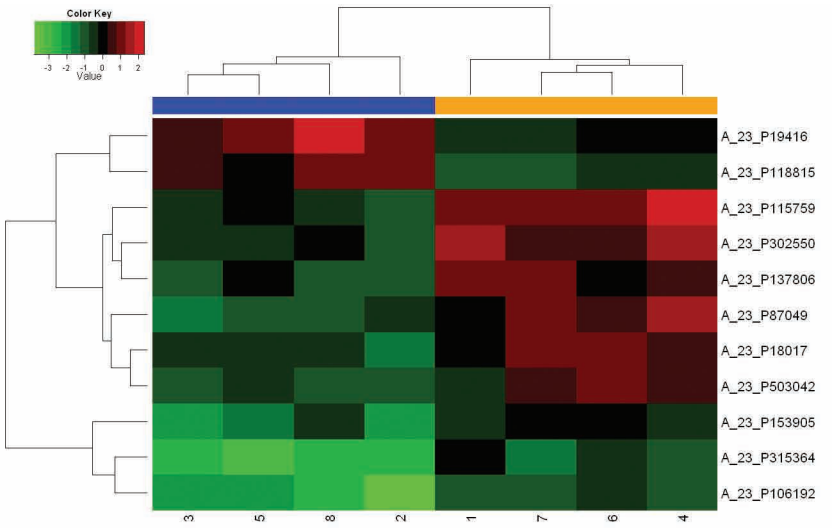

Figure 1. Heatmap of differentially expressed genes in early (upper blue bar) and advanced (upper orange bar) MDS detected by microarrays. The relative gene expression changes are expressed by a gradient intensity of a color scale, as shown in the left corner. The green color indicates down-regulation and red color indicates up-regulation of gene expression in the patient sample. Each row represents a single gene and each column represents a separate patient sample.

The Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.nciferf.gov./) was used for identification of biological enrichment themes [19].

Expression analysis by qRT-PCR. Gene expression of *ADAM8*, *BIRC5* and *MPL* was validated by qRT-PCR. RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega). Expression level of B-2-microglobulin gene (*B2M*) was used as an endogenous control for normalization. Validated TaqMan Assay for *ADAM8*, *B2M* and *MPL* (Applied Biosystems) was adopted. *BIRC5* primer and probe sequences were as follows: forward primer ATGGGTGCCCCGACGTT; reverse primer AGCCTCGGCCATCCGCT; probe 6FAM-CCCCTGCCTGGCAGCCCTT XTC p. Each sample was performed in duplicate using Rotor-Gene 3000 instrument (Corbett Research). The thermal cycling program started at 95°C for 5min, followed by 45 cycles at 95°C for 20sec and at 60°C for 1min. The fold changes of gene expression were calculated using ddC_T method, where $ddC_T = (C_{T,\text{target gene}} - C_{T,B2M})_{\text{patient sample}} - (C_{T,\text{target gene}} - C_{T,B2M})_{\text{control sample}}$ [20].

Results

Microarray assay. We analyzed gene expression profiles in CD34+ cells of 8 MDS patients using Human 1A Agilent arrays and performed comparative analysis of the profiles between early and advanced MDS. Out of the 22 575 tested targets, 286 genes showed significant differential expression ($p<0.01$), including 150 genes with lower expression and 136 genes with higher expression in early MDS compared to advanced stages. DAVID software was used to annotate genes according to gene ontology (GO) and to identify enriched biological processes separately for up-regulated and down-regulated genes. Only the most representative biological processes with the lowest p-values were included in the Table 2.

Clustering analysis clearly discriminated early from advanced MDS patients based on the expression levels of 286 differently expressed genes (data not shown). Further, all three RCMD patients clustered together while RARS patient was an outlying sample within early MDS group. In advanced MDS, patients with RAEB-2 clustered closer to each other then to the rest two patients (RAEB 1-2 and RAEB in transformation to AML).

Table 2. List of enriched biological processes in the subsets of differentially expressed genes between early and advanced MDS patients. The most representative biological processes with the lowest p-value are included.

Gene Ontology (GO)	Count	P-value	Fold enrichment
Genes with lower expression in early MDS			
GO:0007165~Signal transduction	46	6.3×10^{-04}	1.6
GO:0048519~Negative regulation of biological process	21	2.1×10^{-03}	2.1
GO:0007154~Cell communication	47	3.0×10^{-03}	1.5
GO:0007242~Intracellular signaling cascade	23	4.0×10^{-03}	1.9
GO:0006950~Response to stress	18	6.9×10^{-03}	2.0
Genes with higher expression in early MDS			
GO:0006260~DNA replication	12	3.5×10^{-07}	7.8
GO:0000087~M phase of mitotic cell cycle	11	7.1×10^{-06}	6.4
GO:0006974~Response to DNA damage stimulus	10	3.7×10^{-04}	4.5
GO:0006281~DNA repair	8	2.1×10^{-03}	4.4

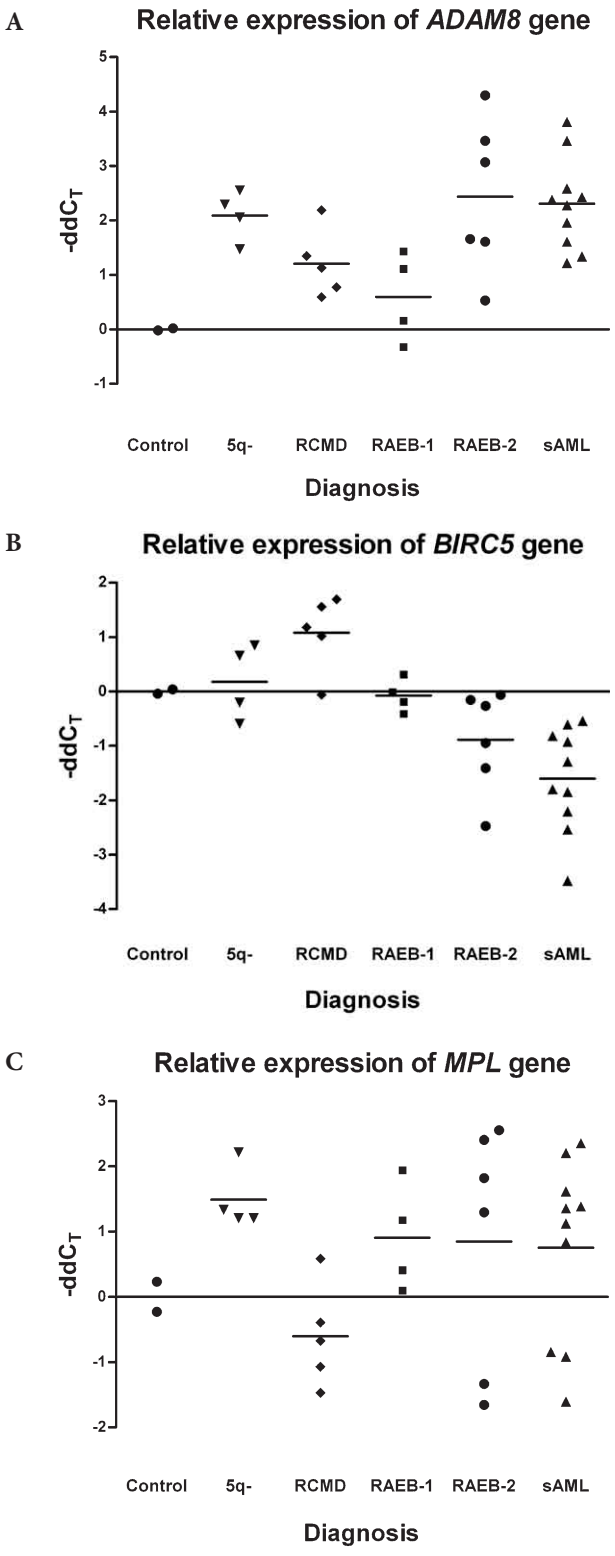

Figure 2. Gene expression of *ADAM8, BIRC5* and *MPL* in particular subtypes of MDS. Gene expression was determined by qRT-PCR and relative fold changes of expression were calculated by $-ddC_T$ method. Each spot represents one patient and the horizontal line in each column marks the mean expression in particular group of patients.

Eleven genes with mean difference of $\log_2$ expression ≥1.3 (representing approximately 2.5 fold real expression difference) between the defined groups were filtered out for further observation. Figure 1 illustrates relative gene expressions for each sample detected by microarrays. Descriptive statistics and gene ontology terms for all 11 genes are listed in the Table 3. Interestingly, patient no. 5 (with RARS) showed completely different expression pattern in *BIRC5*, *ADAM8* and *MPL* genes in comparison to the three other patients with RCMD in the same early MDS category. We detected increased *BIRC5* expression and decreased *ADAM8* and *MPL* expression in RCMD patients, while no expression change was found in RARS patient. This finding underlined different character of RARS and on the other hand the uniformity of RCMD.

Validation of expression levels by qRT-PCR. Out of the array data, expression levels of *ADAM8*, *BIRC5* and *MPL* were validated by qRT-PCR in the additional set of 29 MDS samples (Figure 2). Significance of differential expression between particular groups of patients was evaluated by ANOVA (p-value<0.05) and was confirmed for *ADAM8* and *BIRC5*. In *MPL*, ANOVA reached significant p-value if RAEB-1,2 patients were grouped with sAML since it showed similar expression in all three groups.

Moreover, expression levels of those three genes were highly similar in REAB-2 and sAML group, while RAEB-1 group differed from them especially in *ADAM8* and *BIRC5* expression. Four patients with 5q-syndrome showed relatively low variability in expressions of those three genes. In this group, *ADAM8* and *BIRC5* showed intermediate expression compared to RCMD and RAEB-2/sAML. 5q- patients expressed *MPL* as high as RAEB/sAML; however, its expression was significantly lower in RCMD.

Correlation of ADAM8 and MPL expression with patient clinical course. Expression of *ADAM8* and *MPL* was increased in advanced MDS, but few patients showed markedly lower levels of these genes. In four RAEB-2/sAML patients, we observed low levels of *MPL* as well as *ADAM8* expression. We hypothesized that these patients had some specific clinical features; however, we did not found any association of low gene expression with clinical course.

In contrast, 5 patients (RAEB-2 and sAML) with the highest *MPL* expression showed also high level of *ADAM8* expression with exception of one patient. Those four patients showed disease progression and increased BM blast proportion. Three patients were in RAEB-2 progression associated with BM blast accumulation at the time of analysis. For the last patient increase of BM blast percentage from 18.6% up to 27.4% was observed within one month.

*Correlation of ADAM8, BIRC5 and MPL expression with blast percentage.*BM blast percentage is known to be prognostic marker in MDS. Therefore, we evaluated association of *ADAM8*, *BIRC5* and *MPL* expression with BM blast percentage using Spearman correlation. Information about blast proportion was available for all patients with exception of one RCMD patient. Negative correlation between *BIRC5* expres-

Table 3. Descriptive statistics and Gene Ontology of 11 differentially expressed genes between early and advanced MDS. SD=standard deviation. GO=Gene Ontology

Accession No.	Gene Symbol	GeneName	GO Biological Process	Early MDS		Advanced MDS	
				Mean	SD	Mean	SD
NM_130782	*RGS18*	regulator of G-protein signalling 18	negative regulation of signal transduction (GO:0009968); regulation of G-protein coupled receptor protein signaling pathway (GO:0008277)	-0.49	0.43	0.99	0.55
NM_005373	*MPL*	myeloproliferative leukemia virus oncogene	cell surface receptor linked signal transduction (GO:0007166); cell proliferation (GO:0008283)	-0.61	0.46	0.74	0.49
NM_001109	*ADAM8*	ADAM metallopeptidase domain 8	cell-cell adhesion (GO:0016337); proteolysis (GO:0006508)	-0.50	0.38	1.38	0.62
NM_144503	*F11R*	F11 receptor	inflammatory response (GO:0006954)	-0.91	0.14	0.44	0.54
NM_001870	*CPA3*	carboxypeptidase A3 (mast cell)	proteolysis (GO:0006508)	-0.80	0.49	0.55	0.35
NM_003105	*SORL1*	sortilin-related receptor, L(DLR class) A repeats-containing	receptor-mediated endocytosis (GO:0006898)	-1.07	0.45	0.72	0.69
NM_002089	*CXCL2*	chemokine (C-X-C motif) ligand 2	inflammatory response (GO:0006954); chemotaxis (GO:0006935)	-2.69	0.18	-0.82	0.54
NM_005252	*FOS*	v-fos FBJ murine osteosarcoma viral oncogene homolog	regulation of transcription from RNA polymerase II promoter (GO:0006357); DNA methylation (GO:0006306); inflammatory response (GO:0006954)	-2.71	0.80	-1.01	0.23
NM_004907	*IER2*	immediate early response 2	–	-1.54	0.63	-0.20	0.24
NM_021066	*HIST1H2AJ*	histone cluster 1, H2aj	nucleosome assembly (GO:0006334)	1.29	0.62	-0.28	0.31
NM_001012271	*BIRC5*	baculoviral IAP repeat-containing 5 (survivin)	anti-apoptosis (GO:0006916); apoptosis (GO:0006915); cell cycle (GO:0007049); cytokinesis (GO:0000910); establishment of chromosome localization (GO:0051303); mitosis (GO:0007067); negative regulation of caspase activity (GO:0043154); positive regulation of mitotic cell cycle (GO:0045931); positive regulation of exit from mitosis (GO:0031536); protein complex localization (GO:0031503); spindle checkpoint (GO:0031577)	0.50	0.39	-0.85	0.38

sion and blast proportion was confirmed ($r=-0.83$, $p<0.001$), while for other two genes the correlation was not significant at $\alpha=0.01$.

Since *MPL* is a receptor for trombopoietin (TPOR) and plays a role in megakaryopoiesis, we tested correlation between trombocyte proportion and *MPL* expression but no correlation was found.

Discussion

Microarrays have been repeatedly used in order to understand pathogenesis of MDS at the molecular level and to find appropriate diagnostic/prognostic markers. Several studies were focused on identification of gene set specifically expressed in MDS [22, 25] and detection of differential gene expression between MDS and AML [23]. Due to high heterogeneity of MDS patients, some researchers now pay their attention to an unambiguously defined 5q- syndrome. Thus, unique expression pattern for this subtype in comparison with RA patients and patients with normal karyotype was reported. Hierarchical clustering analysis distinctively discriminated those patients with 5q- syndrome based on the expression profiles [22, 25, 26, 27]. In order to define specific markers of disease progression, expression profiles of MDS patients with respect to different risk of leukemic transformation were compared [21, 24].

In our study, gene expression was analyzed in the BM CD34+ progenitor cells of 4 early (RARS, RCMD) and 4 advanced (RAEB) MDS patients. Within the total set of 286 significantly differently expressed genes, 150 genes showed lower expression and 136 genes higher expression in early MDS compared to advanced stages. We identified most deregulated biological processes using the DAVID database: signal transduction and response to stress for genes down-regulated in early MDS and DNA replication and response to DNA damage stimulus for up-regulated genes in early MDS. Increased DNA replication may reflect higher proliferative activity of hematopoietic progenitors as reported previously [9].

Out of array data, we selected three candidate genes (*ADAM8, BIRC5* and *MPL)* whose expression was validated by qRT-PCR in the larger set of 29 MDS patients.

The higher expression of *ADAM8* was detected in RAEB-2 and sAML patients compared to RCMD and RAEB-1 patients (mean difference=2.9). This gene belongs to ADAMs gene family (a disintegrin and metalloproteinases), which consists of transmembrane glycoproteins involved in essential biological processes: cell-cell fusion, cell-cell interaction and

proteolysis of membrane proteins. Their role in regulation of tumor progression mediated by the regulation of growth factor activities and integrin functions has been reported [28, 29]. We detected three members of this gene family in our set of 286 differently expressed genes: *ADAM8*, *ADAM10*, *ADAM17*, all of them with up-regulation in advanced MDS. They have been reported to be expressed in malignant tumors and their participation in oncogenesis or tumor growth was previously proved [30, 31, 32, 33]. Another three metaloproteases were present in the gene set: *MMP8*, *NPEPPS*, *CPA3*. Similarly, their expression was higher in MDS patients with advanced stages compared to early MDS. These findings underline relevance of *ADAM* genes in the pathogenesis of clonal malignancies.

Further, we observed decreasing expression of *BIRC5* (also known as survivin) from RCMD to RAEB-1,2 and sAML, suggesting association of *BIRC5* expression with disease progression. Similar findings were reported previously in BM mononuclear cells of low-risk and high-risk myelodysplastic patients [40]. In contrast to this study, we did not detect *BIRC5* up-regulation in all MDS patients (only in patients with RCMD and 5q- syndrome) in comparison to healthy controls. This discrepancy could be caused by analyses of different cell populations since in CD34+ cells *BIRC5* is commonly expressed and plays a role in normal adult hematopoiesis [35].

At the protein level, increased expression of BIRC5 was previously found in BM samples of MDS and AML patients. In MDS patients with >5% blast proportion, higher number of BIRC5 positive cells was detected compared to RA or RARS patients, but association of BIRC5 levels with disease progression was not proved [37]. Due to high *BIRC5* expression in RA patients and low or undetectable in aplastic anemia (AA) and chronic myelomonocytic leukemia (CMML), this gene may represent a reliable diagnostic marker of RA [38]. Further, BIRC5 protein expression in BM cells of MDS patients was reported to be lower when overt leukemia developed from MDS [39].

In our study, expression of *MPL* proto-oncogene (*TPOR*) was identified to be decreased in RCMD patients (4/5) and increased in RAEB (8/10) and sAML (7/10) patients compared to healthy controls. This gene encodes transmembrane receptor for thrombopoietin, which is essential in regulation of megakaryogenesis [41]. Increased MPL mRNA/protein level was repeatedly identified in primary AML cases (40-80% of patients) without any obvious association with certain FAB subtype. Similarly increased *MPL* expression was detected in MDS patients, particularly in RAEB and CMML subtypes [42, 43]. Northern blot analysis of MPL protein level in a set of 58 MDS samples resulted in similar findings: no expression was observed for RA and RARS patients while in most RAEB, RAEB-T and CMML patients its expression was detected [44]. Since *MPL* is a member of JAK-STAT signaling pathway, we were interested if any other members of this pathway are present in the set of 286 differentially expressed genes. We found three genes: *JAK1*, *SOCS3* and *PTPN11* showing significantly higher expression in advanced MDS compared to early stages.

Analysis of array data revealed several MDS-related genes that showed deregulation in the studied groups. Gene expression of *FOS* was strongly down-regulated in early MDS patients compared to those of advanced MDS. It is in concordance with previously reported findings of increased expression in AML, demonstrating its potential role of a predictor of blastic transformation [46]. Proto-oncogene *FOS* is involved in regulation of cell proliferation, differentiation and transformation, and in some cases is also associated with apoptotic cell death.

In the set of array data, four members of MAPK signaling pathway were detected: *MAP3K2*, *MAP3K7IP2*, *MAP4K2* and *MAPKAPK3*. Expressions of those four genes were higher in advanced MDS than in early MDS. Three of them are involved in p38 MAPK pathway; moreover, *MAPKAPK3* is directly activated by p38 MAPK. Activation of p38 MAPK was observed in MDS patients and is supposed to inhibit hematopoiesis leading to characteristic cytopenias [47].

In this study, using microarrays we identified the set of 11 genes with mean difference of $\log_2$ expression ≥1.3 (representing approximately 2.5 fold real expression difference) in CD34+ cells between early and advanced MDS patients. Out of them, increased expression of *ADAM8* and *MPL* was confirmed in most of the advanced MDS patients compared to the early MDS patients by qRT-PCR. Further, we detected the opposite trend in *BIRC5* expression with up-regulation in RCMD and down-regulation in RAEB/sAML patients. Our findings demonstrate that the expression levels of these genes are associated with MDS stages and thus they may have potential prognostic significance. Further, we observed deregulation of several genes involved in MAPK and JAK-STAT signaling pathways indicating their possible role in the MDS pathogenesis.

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