Myelodysplastic syndromes with a deletion 5q display a characteristic immunophenotypic profile suitable for diagnostics and response monitoring

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Methods

Patients

The "training cohort" consisted of 156 untreated MDS, 38 patients showed a del(5q), including 26 patients with del(5q) as an isolated aberration or with one additional aberration. The "validation cohort" consisted of 64 MDS [22 with del(5q), 12 showing isolated del(5q) or one additional aberration]. In this cohort 22 MDS [(11 with del(5q)] were diagnosed at the Department of Hematology (VU University Medical Center Amsterdam). Non-clonal cytopenias and healthy age-matched BM samples served as controls (*Online Supplementary Table 1*).

In 18 MDS patients with del(5q), 71 FCM investigations at diagnosis and during the disease course were performed. Within these patients were 10 MDS with del(5q) as a single abnormality and 1 patient with one additional aberration (trisomy 8) included, 7 receiving lenalidomide and 3 azacitidine, a combined treatment within the AZALE trial[1], or an induction therapy with daunorubine plus cytarabine. The further 8 MDS patients showed del(5q) as part of a complex aberrant karyotype and were treated with azacitidine alone (7 patients) or received a combined treatment within the AZALE trial.

This study was performed according to the principles of the Declaration of Helsinki and has received votes from the institutional review boards.

Flow cytometric immunophenotyping

FCM was performed on a FACSCanto II equipped with 3 lasers using FACS-DiVa software (BD Biosciences, San Jose, CA). Instrument set up including fluorescence amplification and compensation was fixed automatically applying FACS-DiVa compensation set up. Flow cytometer performance was checked using CS&T beads (BD Biosciences). In each sample 200000 events were registered. In the VU University Medical Center, Amsterdam, measurements were performed on a FACSCalibur (BD Biosciences).

In both laboratories the applied Boolean gating strategy consisted of the exclusion of doublets and debris, followed by gating of the main populations according to their side scatter (SSC) and CD45 expression. For progenitors with subsequent separation in myeloid (myPC, CD34+CD19-) and lymphoid (lyPC, CD34+CD19+) progenitors CD34 expression and for monocytes CD33/CD36 expression and subsequent backgating to SSC/CD45 was added. Nucleated red cells (NRC) were characterized using CD45, CD235a, and CD71 expression.

We applied published thresholds for abnormal antigen expression as myPC >2.0%, lyPC <5.0%, SSC ratio - granulocytes vs. lymphocytes <6.0.[2,3] All further cut-offs for abnormal expression, mean and 2 standard deviations (SD), have been determined after assessing healthy BM, myPC: CD7 >15.0%, CD45-MFI-ratio (lymphocytes vs. myPC) ≥7.0; granulopoiesis: CD10 ≤25.0%, CD36 >10.0%, CD71 >20.0%;

monopoiesis: CD56 >20.0%; NRC: percentage >8.0, $CD71^{dim}$ >10% of all CD71+ NRC). The threshold for abnormal mean fluorescence intensity (MFI) was set at mean and 2 SD compared to normal BM (e.g. CD15 on granulocytes >5300 in the Dresden and >698 in the Amsterdam lab).

Cytogenetics and molecular genetics

Chromosome preparation, G-banding technique, and karyotyping have been done according to routine cytogenetic procedures. In cases of questionable chromosome morphology results were confirmed by spectral karyotyping (SKY; Applied spectral Imaging, Edingen-Neckarhausen, Germany) or single color FISH with commercial DNA probes. According to the recent cytogenetic classification by Schanz and coworkers[4] MDS presenting with a deletion (5q) were subdivided in MDS with del(5q) as single aberration or with one additional abnormality and in MDS with del(5q) as part of a complex aberrant phenotype.

Evaluation of molecular mutations of *TP53* gene was performed in unselected BM cells using a PCR (exons 4-9) and a DNA based dHPLC (WAVE) analysis by the Munich Leukemia Laboratory, Germany.[5]

Statistical analyses

Data are presented as mean ± SD or as percent patients positive for the analyzed parameter. Non-parametric Mann-Whitney test comparing continuous variables (e.g. SSC ratio) as well as Chi-Square or Fisher's exact test analyzing contingency tables (e.g. % patients with/without increased myPC in del(5q) MDS vs. MDS with normal

karyotype) were performed in the basic assessment of possible differences in the antigen pattern of MDS with del(5q) vs. MDS with NK. As a next step, the most discriminatory immunophenotypic features were evaluated and their discriminatory power has been weighted. Thus, the optimal classifier was constructed using a best subset logistic regression approach with initially potentially influential variables in the training cohort (n=14). The model with the optimal Bayesian information criterion was chosen. The construction of the classifier was performed in the training cohort as well. Receiver operating characteristic analysis (ROC including Youden-Index) have been applied in the following to estimate sensitivity and specificity of del(5q) profile. Therefore, statistical analyses were performed with the statistical computing environment R (a language and environment for computing, R Core Team, Vienna, 2013, version 2.15.1; http://wwwR-project.org) or GraphPad Prism (version 4.0.3, GraphPad Software Inc., San Diego, CA).

Supplementary Tables

Supplementary Table S1. Patient characteristics in the training and validation cohort.

		MDS		non-clonal	healthy BM
		training cohort	validation cohort	cytopenias	
patients	(no.)	156	64[†]	88	50
del(5q)					
isolated[‡]		26	12		
complex		12	10		
NK		88	34		
other karyotypes		30	8		
age (years)[*]	(median; range)	69 (19-84)	72 (26-89)	66 (18-87)	65 (45-82)
gender	(f/m)	56/100	23/41	39/49	24/26
IPSS	(median; range)	0.5 (0.0-3.5)	0.5 (0.0-3.0)		
IPSS-R	(median; range)	3.0 (0.0-10.0)	3.0 (1.0-8.5)		

[†]In this cohort 22 MDS [11 with del(5q)] were diagnosed and investigated with flow cytometry at the Department of Hematology of the VU University Medical Center Amsterdam. [‡]This includes MDS with del(5q) as a single abnormality (19 in the training and 9 in the validation cohort) or with one additional abnormality. *There were no significant differences in gender or age between MDS and non-clonal cytopenias or healthy BM controls. BM, bone marrow; NK, normal karyotype; f, female; m, male.

Supplementary Table S2A. 8-color antibody panel used at Technical University Dresden.

FITC	PE	PerCP5.5	PE-cy7	APC	APC-H7	PacBlue	V500
CD2	**CD13**	**CD34**	**CD10**	**CD117**	**HLA DR**	**CD19**	**CD45**
(MT910)	(L138)	(8G12)	(HI10a)	(104D2)	(L243)	(HD37)	(HI30)
DAKO	BD	BD	BD	BD	BD	DAKO	BD
CD7	**7.1**	**CD34**	**CD123**	**CD5**	**HLA DR**	**CD3**	**CD45**
(Leu-9)	(7.1)		(6H6)	(L17F12)		(UCHT1)	
eBioscience	BC		Biolegend	BD		DAKO	
CD16	**CD13**	**CD34**	**CD64**	**CD15**	**HLA DR**	**CD11b**	**CD45**
(3G8)			(10.1)	(HI98)		(ICRF44)	
BC			BD	BD		Biolegend	
CD14	**CD36**	**CD34**	**CD56**	**CD33**	**HLA DR**	**CD11b**	**CD45**
(M5E2)	(CB38)		(NCAM16.2)	(P67.6)			
BD	BD		BD	BD			
CD235a	**CD36**	**CD34**	**CD117**	**CD71**		**CD105**	**CD45**
(10F7MN)			(104D2)	(L01.1)		(266)	
eBioscience			BD	BD		BD	

CD number, clone, and supplier were provided for each antibody. BD: Becton Dickinson; BC: Beckman Coulter.

Supplementary Table S2B. 4-color panel used at Amsterdam Cancer Center.

FITC	PE	PerCP5.5	PE-cy7
CD16 (DJ130c) DAKO	**CD13** (L138) BD	**CD45** (2D1) BD	**CD11b** (D12) BD
CD34 (8G12) BD	**CD11b** (D12) BD	**CD45**	**HLA-DR** (L243 (G46-6)) BD
CD36 (CLB-IVC7) Sanquin	**CD33** (P67.6) BD	**CD45**	**CD14** (MoP9) BD
CD36	**CD64** (10.1) DAKO	**CD45**	**CD14**
CD15 (MMA) Sanquin	**CD10** (SS2/36) DAKO	**CD45**	**CD34** (8G12) BD
CD34	**CD117** (104D2) BD	**CD45**	**CD13+CD33** (WM15+P67.6) BD
CD5 (DK23) DAKO	**CD19** (SJ25C1) BD	**CD45**	**CD34**
CD2 (MT910) DAKO	**CD56** (My31) BD	**CD45**	**CD34**
CD13 (WM-47) DAKO	**CD7** (M-T701) BD	**CD45**	**CD34**
CD34	**CD123** (9F5) BD	**CD45**	**HLA-DR**
CD71 (Ber-T9) DAKO	**CD235a** (JC159) DAKO	**CD45**	**CD117** (104D2) BD

CD number, clone, and supplier were provided for each antibody. BD, Becton Dickinson.

Supplementary Table S3A. Logistic regression analysis with estimates and significances from the analysis of the final 5-parameter-del(5q)-score.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-23.623	1314.094	-0.018	0.986
% myPC	2.591	0.607	4.266	<0.001
CD45 ratio	18.028	1314.093	0.014	0.989
SSC ratio	2.070	0.552	3.749	<0.001
% CD71 gran	1.477	0.627	2.355	0.019
female gender	1.594	0.532	2.995	0.003

Supplementary Table S3B. ROC table of the validation set from the analysis of the final 5-parameter-del(5q)-score.

	sensitivity	specificity	cut-off
1	0	100	inf
2	27	100	18.0
3	55	95	16.5
4	59	95	16.0
5	77	93	15.0†
6	95	81	14.5
7	100	74	13.5
8	100	50	13.0
9	100	31	11.5
10	100	21	10.0
11	100	19	8.0
12	100	17	6.5
13	100	10	4.5
14	100	2	3.0
15	100	0	0.0

†ROC curve: AUC=0.946; *P*<0.001.

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