CD34+ gene expression profiling of individual children with very severe aplastic anemia indicates a pathogenic role of integrin receptors and the proapoptotic death ligand TRAIL

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Online Supplementary Design and Methods

mRNA amplification and labeling

The 'Two-Cycle Target Labeling Protocol' by Affymetrix (Santa Clara, CA, USA) was modified due to the limited amounts of RNA available. After cDNA synthesis, unlabeled ribonucleotides were used in the first RNA amplification step. This cRNA was reverse transcribed using random primers. T7-oligo(dT) promoter primers were used for second-strand cDNA synthesis to generate a double-stranded cDNA template containing T7 promoter sequences. This cDNA was amplified and labeled using a biotinylated nucleotide/ribonucleotide mix in the second IVT. The concentration of the biotin-modified cRNA was determined using an Agilent 2100 Bioanalyzer® according to the manufacturer's recommendations. After enzymatic fragmentation, all cRNA samples contained fragments between 30 nt to 200 nt with a peak at 100 nt.

Array data analysis

Raw data obtained with the Affymetrix GeneChip U133 Plus 2.0 array passed the quality control procedures implemented in the R-package ArrayQualityMetrics (ver. 2.2.1). The Robust MultiChip Analysis (RMA)[1] was then applied to the 31 samples investigated (13 vSAA, 8 RC and 10 normal controls (C)) to assess differences in gene expression. Moderated t-statistics were calculated as implemented in R-package limma (ver. 2.18.2).[2] Benjamini and Hochberg multiple testing correction was performed to control false discovery rate (FDR).[3]

To compare these gene expression data with 66 previously published[4] samples of healthy controls (N, n=11) and patients with myelodysplastic disease, including RC (n=18), refractory anemia with ringed sideroblasts (RARS, n=19) and refractory anemia with excess blasts (RAEB, n=19), all samples were normalized using RMA. We used Support Vector Machines (SVM)[5] to classify samples into subsets according to their gene expression patterns.

Detection of platelet reactive antibodies by platelet adhesion immunofluorescence test (PAIF)

A panel of phenotyped adherent platelets were incubated with sera from vSAA patients as previously described.[6] Platelet-reactive antibodies were then detected employing an FITC-conjugated anti-human antibody and analyzed by fluorescence microscopy.

Detection of platelet reactive antibodies by monoclonal antibody-specific immobilization of platelet antigens (MAIPA)

Patient sera were also tested by monoclonal antibody-specific immobilization of platelet antigens (MAIPA).[7] A panel of monoclonal antibodies (Gi5, SZ22, FMC25, Gi9, Gi18, D2, B1G6) specific for GPIIb/IIIa, GPIIb, GPIb/IX, GPIa/IIa, CD31, CD109 and β2-microglobulin of HLA-class I antigen, respectively, were used as capture antibodies. Autoreactive antibodies bound to immobilized glycoproteins were detected by anti-human antibodies coupled to horse radish peroxidase. The enzymatic reaction was measured at 490 nm with a microtiter plate reader (Tekan, Greilsheim, Germany). An absorbance of more than 0.2 was considered reactive. Reproducibility was controlled using the international standard HPA-1a antibody (NIBSC 03/152).

References

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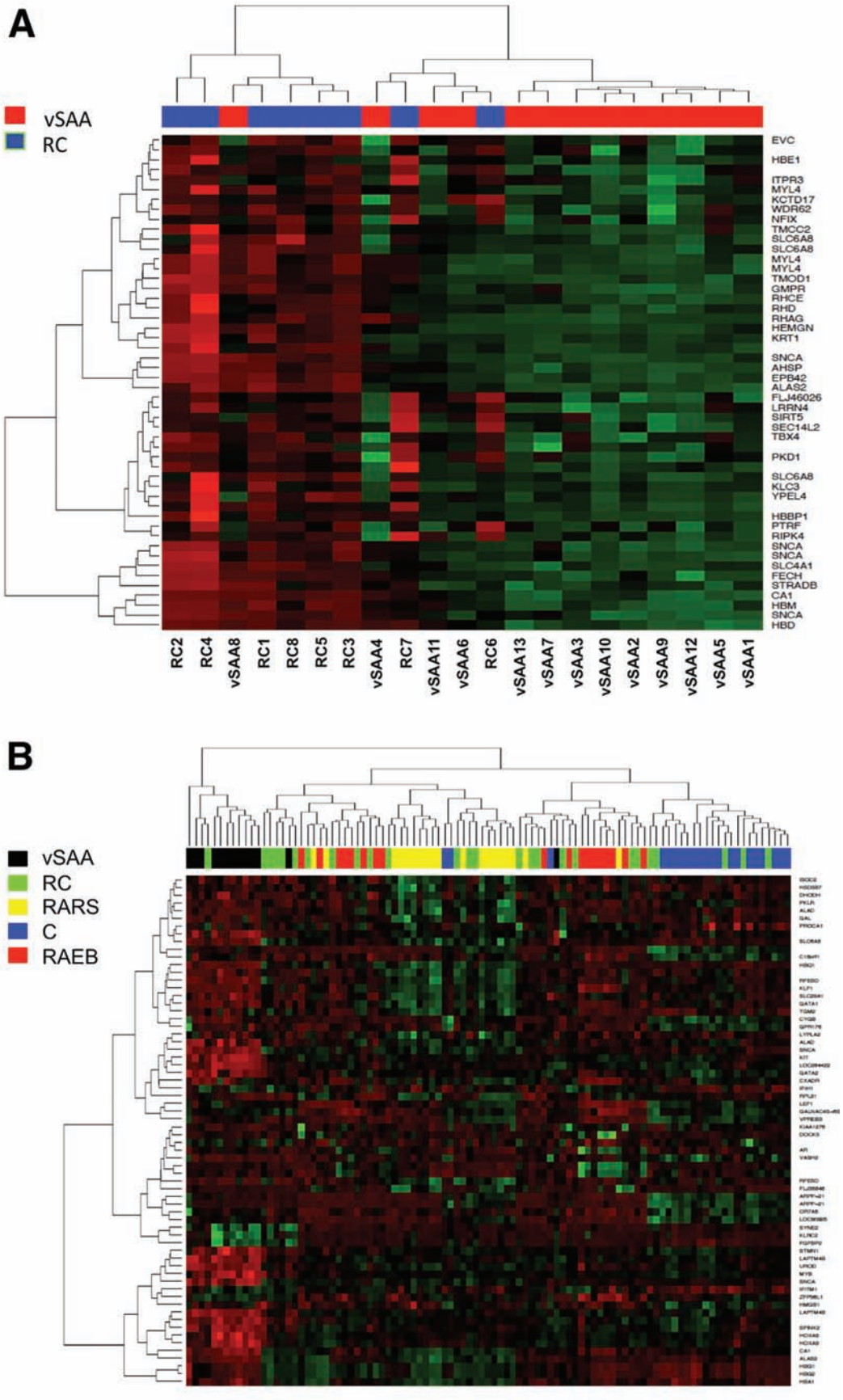
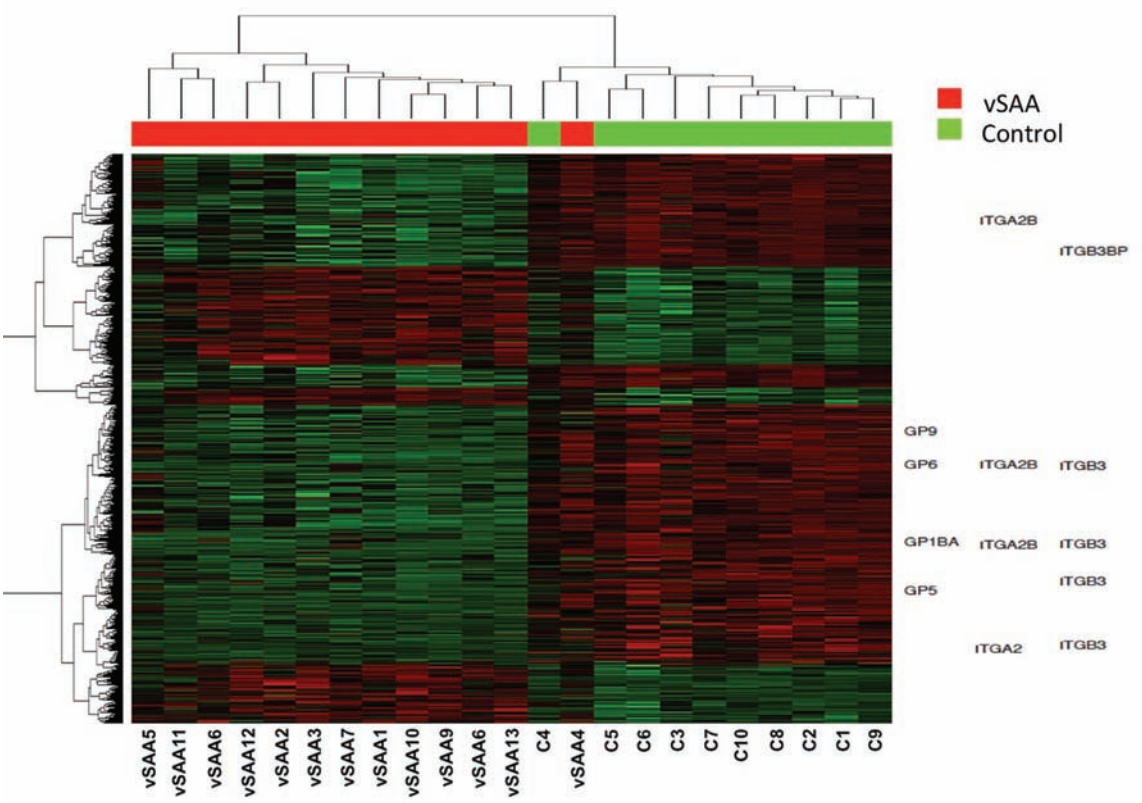
Online Supplementary Table S1. SEE PDF FILE

Online Supplementary Table S2. List of up-regulated genes in the vSAA patient cohort involved in apoptotic and autophagic signaling.

Function	Gene Symbol	GenBank#	logFC	adj. *P*-value
Granzymes and Perforin	GZMA	NM_006144	3,53	<0,001
	GZMB	BC030195	1.88	0,023
	GZMH	M72150	2.61	0,002
	GZMK	BC035802	2.19	0,003
	PRF1	BC047695	2.55	0,004
Extrinsic DR-pathway	FAS	U89101	0.59	0.002
	TNFSF10	U37518	1.6	0.031
	TNFRSF10C	AF012536	1.6	0.027
	TRADD	L41690	0.72	0.004
	TNFSF12	AF030099	0.66	0,023
	TRAF1	AK090468	0.8	0,036
	TRAF3	U21092	1.56	<0,001
	TRAF3IP1	AF230877	0.38	0,020
	BIRC3	L49432	1.33	0,031
	TNFAIP3	M59465	0.96	0,011
Intrinsic mitochondrial pathway	BCL2L11	AF032456	2.12	0,001
Autophagy	BECN1	AF077301	0.72	0,046
	ATG16L2	AK024423	0.86	0,025



Online Supplementary Figure S1. Gene expression profiling allows differentiation between the clinically similar disease states vSAA and MDS. (A) Hierarchical clustering of 13 pediatric patients with vSAA and 8 pediatric patients with newly diagnosed refractory anemia. (B) Hierarchical clustering of the 13 samples of vSAA patients, 21 samples of healthy controls (N), and 55 samples of patients with myelodysplastic disease, including refractory cytopenia (RC), refractory anemia with ringed sideroblasts (RARS) and refractory anemia with excess blasts (RAEB). Hierarchical clustering of 66 most differentially expressed probes between the five subgroups. Each row corresponds to a probe and each column to a patient or control subject. Green is used for high intensities and red for low.



Online Supplementary Figure S2. mRNA expression of platelet membrane bound glycoproteins is down-regulated in surviving CD34$^+$ cells from vSAA patients. Heatmap of samples from 13 vSAA patients and 10 normal control persons (C) analyzed as in Table 1 and Figure 1. Hierarchical clustering of the 1,500 top ranked probesets is shown, containing all integrin and platelet glycoprotein probes (annotated).

Online Supplementary Table S3. Down-regulated platelet membrane-bound glycoproteins in 13 vSAA samples.

Gene symbol	Synonym	Complex	GenBank# RefSeq#	logFC	adj. *P* value	Gene Phenotype Relationship	Auto-antibodies
GP5	GP V	GPIB/GPIX/ GPV	L11238	-2.89 -0.61	6.4 e-07 0.044	Gold-induced thrombocytopenia	Varicella zoster or gold-induced TP
GP6	GP VI	-	AB035073	-1.25	0,004	Amegakaryocytic thrombocytopenia	GPVI deficiency
GP9	GP IX	GPIB/GPIX/ GPV	NM_000174	-1.53	0,003	Bernard-Soulier Syndrome	Drug-induced TP
GP1BA	GPIb α	GPIB/GPIX/ GPV	NM_000173	-3.11	3.9 e-06	Bernard-Soulier Syndrome	Chronic ITP, HCV, lupus anticoagulant
ITGA2B	integrin α-2B; GPIIb	GPIIb/IIIa	NM_000419	-3.77 -2.46 -4.23	2.0 e-05 7.8 e-05 <0.001	Glanzmann thrombasthenia	NAIT
ITGA2	integrin α-2; GPIa	GPIa/IIa	NM_002203	-1.44	0,001	Glycoprotein Ia deficiency	NAIT, SLE
ITGB3	integrin β-3; GPIIIa	GPIIb/IIIa	NM_000212	-4.02 -1.8 -2.24 -1.28	3.2 e-07 8.5 e-07 2.3 e-05 <0.001	Glanzmann thrombasthenia	

logFC: fold change compared to 10 normal controls.