SBDS-deficient cells undergo accelerated apoptosis through the Fas-pathway

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Supplemental Data

Oligonucleotide microarray methods and statistics

The chip contains over 47,000 transcripts, including 38,500 well characterized human genes. Total RNA was extracted from post-Ficoll marrow mononuclear cells using TRIzol (Invitrogen, Carlsbad, CA, USA) as previously described[1] followed by precipitatation in NaOAC (Sigma, St. Louis, MO, USA) and cold ethanol (Commercial Alcohols Inc., Canada). RNA integrity was assessed qualitatively on an Agilent 2100 Bioanalyzer. RNA labelling, hybridization, scanning and data analysis were performed according to the Affymetrix protocol (Affymetrix Inc.). In brief, 2.5 μg of total RNA from each sample was reverse transcribed using T7-Oligo(dT) promoter primers, followed by second-strand cDNA synthesis, in-vitro transcription by T7 RNA polymerase and biotinylated nucleotides to produce biotin-labeled complementary RNA (cRNA). The biotinylated targets were cleaned up, fragmented and 15 μg was used for hybridization with the GeneChip. The hybridization, staining and destaining steps were carried out using the Fluidics Station 400 (Affymetrix Inc.). Scanning was carried out with the GeneChip® Scanner 3000 (Affymetrix Inc.). Hybridization image was obtained and processed in GeneChip Operating Software, and all experiments were scaled to a target intensity of 150 in their signal values. Gene expression signal, detection call, signal log ratio and change call were calculated in GeneChip Operating Software by the statistical algorithm published previously by Affymetrix.[2,3]

Data analysis was performed in R statistical computing language, release 2.0.1.[4] Signal quantification and normalization were performed using robust multichip analysis (RMA) in the Bioconductor affy package (*http://www.bioconductor.org*).[5] Test of differentially expressed genes was carried out using linear models and Empirical Bayes analysis (limma package in *http://www.bioconductor.org*).[6] Genes were ranked based on an empirical Bayes log odds of differential expression (B statistics in limma) and the false discovery rate (FDR) adjusted p values were also used to judge level of significance. Moderated T statistic values were used to screen apoptosis-related genes. With the traditional t–test, the variance of each gene is estimated individually, which works well when there are sufficient replicates. However, with few replicates per group (typical of many microarray experiments), the gene-specific variance estimates can be unstable. In a moderated t–statistic, which has been used in this paper, the estimated gene–specific variance s^2_g is augmented with s^2_0, a global variance estimator obtained from pooling all genes. This approach gives an interpolation between the traditional t–statistic and a fold–change criterion. The moderated T statistic for each gene is given by

$$T_g = \frac{\bar{x}_{g,sds} - \bar{x}_{g,control}}{\sqrt{\mu s_g^2 + \lambda s_0^2}}$$

where the numerator quantifies the fold change and the denominator gives a stable estimate of standard error for each gene g by shrinking the estimated sample variances towards a pooled estimate. This moderated t-test is more robust when the number of arrays is small. An analysis solely based on fold change is not recommended because it does not allow an assessment of significance of observed differences in the presence of biological and experimental variation.

References

1. Rujkijyanont P, Beyene J, Wei K, Khan F, Dror Y. Leukaemia-related gene expression in bone marrow cells from patients with the preleukaemic disorder Shwachman-Diamond syndrome. Br J Haematol 2007;137:537-44.
2. Liu WM, Mei R, Di X, Ryder TB, Hubbell E, Dee S, et al. Analysis of high density expression microarrays with signed-rank call algorithms. Bioinformatics. 2002;18(12):1593-9.
3. Hubbell E, Liu WM, Mei R. Robust estimators for expression analysis. Bioinformatics 2002;18:1585-92.
4. Ihaka R. A language for data analysis and graphics. J Comput Graph Stat. 1996;5:299-314.
5. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249-64.
6. Smyth G. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Statistical applications and molecular biology. Stat Appl Genet Mol Biol 2004;Article3. Epub 2004 Feb 12.

Table 1. Characteristics of the patients who were studied for microarray analysis.

UPN	*Age at testing (Years)/Gender*	*SBDS Mutational Analysis*	*Neutropenia/ Anemia/ Thrombocytopenia*	*Bone Marrow hypocellularity/ Abnormal cytogenetics*
9	19.5/F	183-184TA>CT /258+2T>C	N, A, T	Yes/No
12	14.3/M	258+2T>C / 258+2T>C+183-184TA>CT	N, T	Yes/No
14	20/M	258+2T>C / 260T>G	N	Yes/No
20	11.3/M	258+2T>C / 258+2T>C+183-184TA>CT	N, T	Yes/No
21	24/M	183-184TA>CT / 258+2T>C	N, A, T	Yes/No
37	32.9/M	ND	N, T	Yes/No
55	3/F	258+2T>C / 258+2T>C+183-184TA>CT	None	Yes/No
60	22/F	ND	N, A, T	Yes/No
88	8.3/M	258+2T>C / 120delG	N	Yes/No

All patients also had pancreatic dysfunction. A: anemia (hemoglobin concentration < 2 standard deviations below mean, adjusted for age (Dallman, et al, 1977); N: neutropenia (neutrophil concentration < 1.5 ? 109/L); n, normal; ND: not done; T: thrombocytopenia (platelet count < 150x0^9/L); UPN, unique patient number.

Table 2. Apoptosis-related genes with prominent increased expression in SDS marrow cells (T value of >1.9).

Gene Symbol	*U133 Plus 2.0 probe set*	*GenBank reference*	*UniGene reference*	*M*	*T*
TNF Ligand family					
TNFSF14	207907_at	NM_003807	Hs.129708	0.54	3.68
TNFSF10 (TRAIL)	202687_s_at	U57059	Hs.83429	1.10	3.03
TNFSF6 (FASL)	211333_s_at	AF288573.1	Hs.2007	0.47	2.93
TNF receptor family					
TNFRSF4	214228_x_at	AJ277151	Hs.129780	0.44	3.38
TNFRSF18	224553_s_at	AF117297	Hs.212680	0.51	3.02
TNFRSF1A	207643_s_at	NM_001065	Hs.159	0.42	2.07
TNFRSF12	216042_at	AI275938	Hs.180338	0.28	2.02
TNFRSF6 (FAS)	204781_s_at	NM_000043	Hs.82359	0.33	1.95
Mitochondrial family					
BCL2L1 (BCL-X)*	215037_s_at	U72398	Hs.305890	1.63	5.68
BOK	221454_at	NM_014204	Hs.307355	0.31	2.29
Caspase family					
CASP10	210955_at	U86214.1	Hs.5353	0.35	2.98
CASP14	231722_at	NM_012114	Hs.248226	0.42	2.42
TRAF family					
TANK	207616_s_at	NM_004180.1	Hs.146847	0.64	4.08
P53 and ATM pathway					
ATM	1554631_at	BC007023	Hs.194382	0.73	2.80
Fas-related transcription factors not included above					
EPHB2 (ERK)	234158_at	AK025173	Hs.306776	0.35	2.08

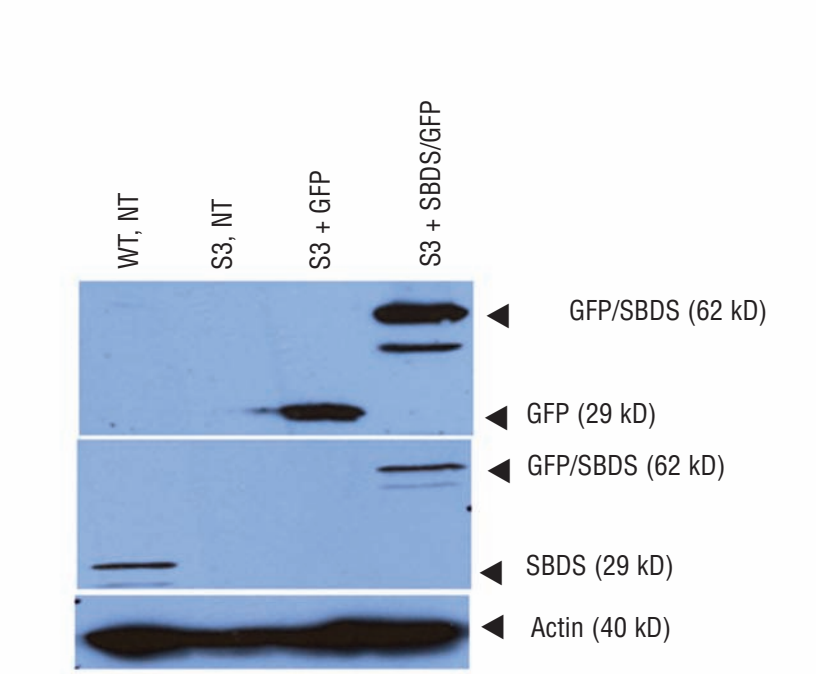
**The probe recognizes both pro-survival isoform Bcl-XL and the pro-apoptosis isoform Bcl-XS (See text). neutropenia (neutrophil concentration < 1.5x10^9/L); n, normal; ND,: not done; T: thrombocytopenia (platelet count < 150x10^9/L); UPN: unique patient number.*

Table 3. Apoptosis-related genes with prominent decreased expression in SDS marrow cells (T value of < -1.9).

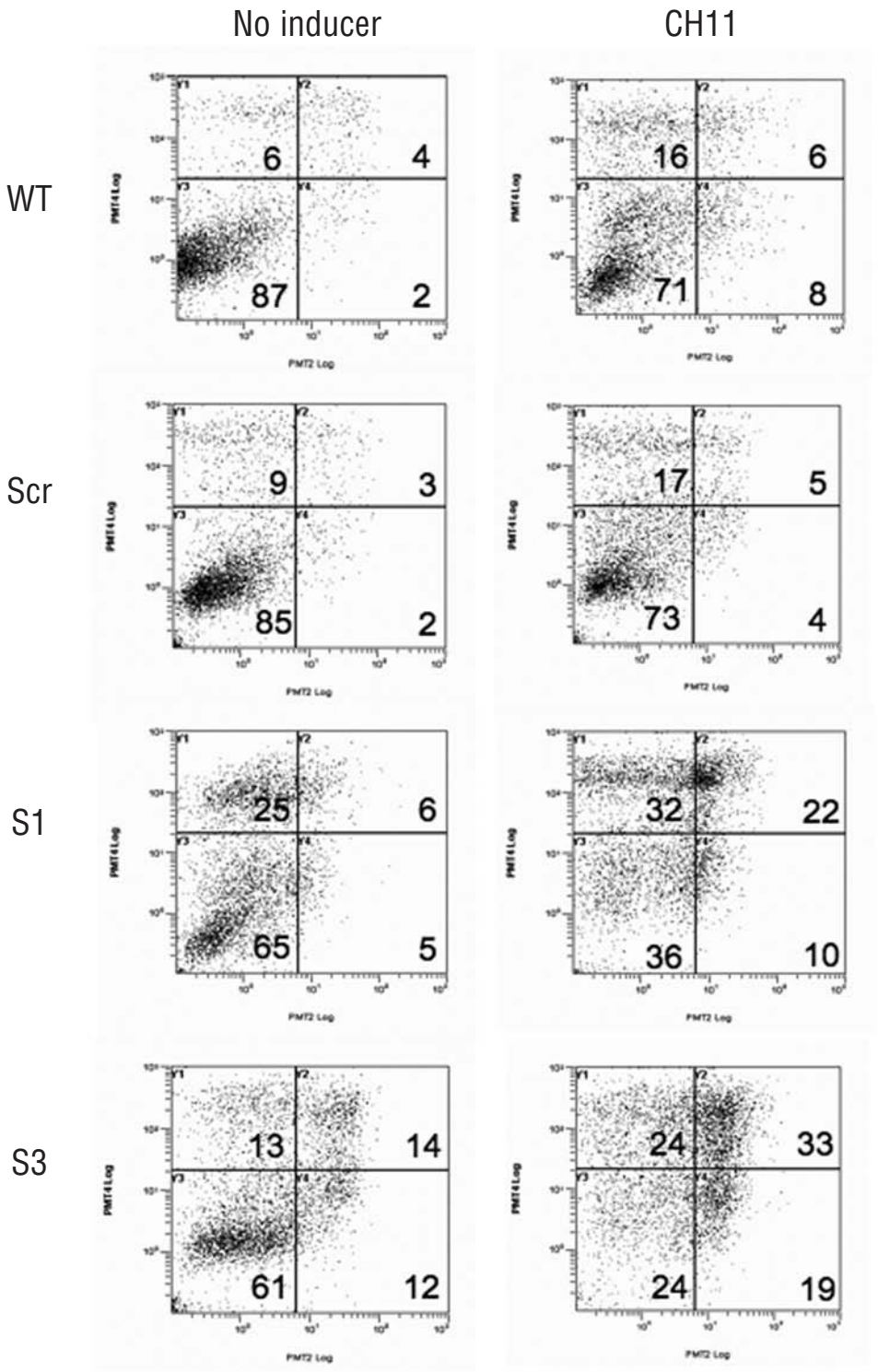
Gene Symbol	*U133 Plus 2.0 probe set*	*GenBank reference*	*UniGene reference*	*M*	*T*
TNF ligand family					
TNFSF4	207426_s_at	NM_003326.1	Hs.181097	-0.72	-3.25
Mitochondrial family					
MCL-1	214057_at	H71805	Hs.86386	-0.79	-3.39
HRK	206864_s_at	NM_003806	Hs.87247	-0.74	-3.05
BCL-2	203684_s_at	M13994.1	Hs.79241	-0.21	-2.02
BLK	206255_at	NM_001715	Hs.2243	-0.80	-1.98
Caspase family					
CASP6	209790_s_at	BC000305	Hs.3280	-0.49	-1.99
IAP family					
BIRC5	1555826_at	BQ021146	Hs.1578	-0.70	-2.73
CARD family					
RIPK2	209545_s_at	AF064824	Hs.103755	-0.71	-2.44
CIDE Domain family					
DFFA	203277_at	NM_004401.1	Hs.105658	-0.29	-2.42
P53 and ATM pathway					
TP53	201746_at	NM_000546	Hs.1846	-0.55	-3.44
MDM2	233436_at	AK022122.1	Hs.287481	-0.50	-3.31
Fas-related transcription factors not included above					
GABPB2	236164_at	AI796858	Hs.122974	-0.71	-3.91
JUN	201466_s_at	NM_002228	Hs.78465	-1.36	-3.03
NFkB2	207535_s_at	NM_002502	Hs.73090	-0.61	-2.6

Table 4. Apoptosis-related genes with differential expression by T value of between 1.9 and -1.9 in the SDS marrow cells.

Gene Symbol	*U133 Plus 2.0 probe set*	*GenBank reference*	*UniGene reference*	*M*	*T*
TNF Ligand family					
LTA (TNF-B)	234897_s_at	H10318	Hs.247478	-0.05	-0.27
LTB	207339_s_at	NM_002341.1	Hs.890	0.09	0.31
TNFSF5	207892_at	NM_000594.1	Hs.241570	0.02	0.10
TNFSF7	206508_at	NM_001252	Hs.99899	0.19	1.55
TNFSF8	207216_at	NM_001244.1	Hs.1313	0.11	0.83
TNFSF9	206907_at	NM_003811.1	Hs.1524	0.08	0.38
TNFSF11	210643_at	U57059.1	Hs.83429	0.001	0.01
TNFSF12	205611_at	NM_003809.1	Hs.26401	0.05	0.35
TNFSF13	229326_at	BE675173	Hs.54673	0.12	0.86
TNF Receptor Family					
LTBR	203005_at	NM_002342.1	Hs.1116	-0.001	-0.01
TNFRSF1B	203508_at	NM_001066.1	Hs.256278	0.16	0.62
TNFRSF5	215346_at	BF664114	Hs.25648	-0.09	-0.59
TNFRSF7	206150_at	NM_001242.1	Hs.180841	0.08	0.31
TNFRSF8	206729_at	NM_001243	Hs.1314	0.19	1.84
TNFRSF9	207536_s_at	NM_001561.2	Hs.73895	0.18	1.13
TNFRSF10A	1552648_a_at	NM_003844.2	Hs.249190	0.13	0.97
TNFRSF10B	209295_at	AF016266	Hs.51233	0.10	0.37
TNFRSF10C	206222_at	NM_003841.1	Hs.119684	-0.06	-0.22
TNFRSF14	209354_at	BC002794.1	Hs.279899	0.04	0.29
Mitochondrial Family					
BAX	208478_s_at	NM_004324	Hs.159428	0.07	0.28
BAK1	203728_at	NM_001188.1	Hs.93213	0.03	0.30
BCL2A1	205681_at	NM_004049.1	Hs.227817	-0.24	-0.62
BCL2L11	208536_s_at	NM_006538.1	Hs.202657	0.09	0.24
BCL2L2	1555140_a_at	BC021198	Hs2.75244	0.12	0.84
BNIP3	201849_at	NM_004052	Hs.79428	-0.16	-0.59
BIK	213132_s_at	AL022237	Hs.7436	0.04	0.27
Caspase Family					
CASP1	206011_at	AI719655	Hs.2490	0.60	1.89
CASP2	208050_s_at	NM_001224.1	Hs.108131	-0.03	-0.15
CASP3	202763_at	NM_004346.1	Hs.74552	-0.23	-1.08
CASP4	209310_s_at	U25804.1	Hs.74122	-0.11	-0.42
CASP5	207500_at	NM_004347.1	Hs.3257	0.11	1.06
CASP7	207181_s_at	NM_001227.1	Hs.9216	-0.03	-0.14
CASP8	207686_s_at	NM_001228	Hs.19949	-0.07	-0.22
CASP9	210775_x_at	AB015653.1	Hs.100641	0.05	0.35
CASP13	208340_at	NM_003723	Hs.137587	0.14	1.33
IAP Family					
BIRC1	204861_s_at	NM_004536.1	Hs.79019	-0.04	-0.13
BIRC2	202076_at	NM_001166.2	Hs.289107	0.16	1.04
BIRC4 (XIAP)	206537_at	NM_001167.1	Hs.172777	-0.05	-0.46
BIRC6	233093_s_at	AK023788	Hs.250646	0.02	0.10
TRAF Family:					
TRAF1	205599_at	NM_005658.1	Hs.2134	0.03	0.21
TRAF2	204413_at	NM_021138	Hs.200526	-0.09	-0.76
TRAF3	208315_x_at	NM_003300.1	Hs.297660	-0.07	-0.54
TRAF4	211899_s_at	AF082185	Hs.8375	-0.02	-0.12
TRAF5	204352_at	NM_004619	Hs.29736	-0.14	-0.46
TRAF6	205558_at	NM_004620.1	Hs.90957	-0.32	-1.82
CARD Family					
APAF1	211553_x_at	AF248734	Hs.77579	0.04	0.18
ASC	221666_s_at	BC004470.1	Hs.71869	-0.11	-0.54
BCL10	205263_at	AF082283.1	Hs.193516	-0.12	-0.63
NOD1	221073_s_at	NM_006092	Hs.19405	0.06	0.70
Death Effector Domain Family					
CFLAR (C-FLIP)	214486_x_at	AF041459	Hs.195175	-0.01	-0.08
FADD	202535_at	NM_003824.1	Hs.86131	-0.01	-0.08
LOC51283	218056_at	NM_016561.1	Hs.168159	0.05	0.25
CIDE Domain Family:					
CIDEA	221295_at	NM_001279	Hs.249129	0.15	1.46
CIDEB	221188_s_at	NM_014430.1	Hs.288835	-0.12	-0.90
DFFB	206752_s_at	NM_004402.1	Hs.133089	-0.36	-1.55
p53 and ATM pathway					
CHEK1	205393_s_at	NM_001274	Hs.20295	-0.21	-1.01
GADD45A	203725_at	NM_001924.2	Hs.80409	-0.15	-0.63
RPA3	209507_at	BC005264.1	Hs.1608	-0.07	-0.51
Fas-related transcription factors not included above					
FOS	209189_at	BC004490	Hs.25647	0.12	0.40
GABPA	227428_at	BE876628	Hs.5905	0.02	0.09
NFkB1	209239_at	M55643	Hs.83428	0.06	0.35



Supplemental Figure 1. Verification of the chicken anti-Sbds antibody. To verify the specificity and sensitivity of our chicken anti-human Sbds antibody, HeLa cells were transfected with a pcDNA3.1 vector containing either GFP or GFP-SBDS fusion gene. After transfection, cell lysates were prepared and analyzed by western blotting. The first two lanes (from the left) are from HeLa/WT (WT) or HeLa/shSBDS-3 (S3) cells without transfection (NT). The right two lanes are from HeLa/shSBDS-3 cells transfected with a vector containing either GFP alone or GFP-SBDS fusion gene. The upper film shows expression of GFP only in the cells transfected GFP or GFP/SBDS. The middle film shows SBDS expression using the chicken and-human SBDS. Positive bands are seen only in lysates from HeLa/WT or from HeLa/shSBDS-3 in which the SBDS was re-introduced. The lower film shows actin loading control.



Supplemental Figure 2. Apoptosis analysis of SBDS-knockdown and control cells by Annexin V and propidium iodide staining. Cells were incubated for 24 hours with or without activating Fas antibody (CH-11, 0.02μg/mL), then harvested and stained with annexin V and propidium iodide. Note that detection of apoptosis by annexin V and propidium iodide staining is highly sensitive. Therefore, the percentages of early (right lower quadrants) and late apoptosis (upper two quadrants) are higher than the numbers of apoptotic cells detected by DNA content analysis (*Manuscript Figures 2A, 2B and 3C*).