SUPPLEMENTAL MATERIAL

Title: M**ultiparametric Flow Cytometry highlights B7-H3 as a novel diagnostic/therapeutic target in GD2 neg/low Neuroblastoma variants**

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METHODS

Cytomorphological Analysis (CA)

BM aspirates smears were stained with May Grunwald-Giemsa and analyzed using Light Microscopy (LM). According to the amount of clustered tumor cells and small round blue cells with high nucleus/cytoplasm ratio in 3 BM smears analyzed, infiltration's score was assigned as following: 1+ = 1-2 clumps/smear; 2+ = >2-3 clumps/smear; 3+ = massive, leukemic-like infiltration.

Immunohistochemical Analysis

For all cases primary tumors and, in some cases, BM trephine biopsies were analyzed by means of standard histological methods (formalin-fixed, paraffin-embedded samples, hematoxylin/eosin- stained step sections). On BM trephines Immune-histochemical stains were routinely performed with the following antibodies: anti-CD56, anti-Neuron Specific Enolase (NSE), anti-chromogranin (Roche Italia, S.p.A.) and Phoxo-2B (Abcam, Cambridge, UK)(11).

Monoclonal antibodies and reagents

M5B14 mAb (IgM, mouse anti-B7-H3), A6/220 (IgM, mouse anti-CD56) and A6/136 (IgM mouse anti-HLA-I) were produced in our lab(6). Anti-GD2 (14.G2a, IgG2A) and anti-CD45 mAbs were purchased from BD Bioscience PharMingen (San Diego, CA). Anti-CD45-V500-C (2D1 clone), anti-CD56-PE-CY7 (NCAM16.2 clone), anti-GD2 AlexaFluor647 (14.G2a clone), anti-CD276 BV421 (7-517 clone), anti-CD73 PerCP-Cy5.5 (AD2 Clone), anti-CD13 PerCP-Cy5.5 (WM15 Clone), anti-CD105 PerCP-Cy5.5 (266 Clone) and anti-CD90 PerCP-Cy5.5 (5E10 Clone) were purchased from BD Biosciences (San Diego, CA, USA).
Syto™16 Green Fluorescence nucleic was purchased from Life technologies.

Neuroblastoma cell lines

SH-SY5Y cell line was purchased from Banca Biologica and Cell Factory (IRCCS Azienda Ospedaliera Universitaria San Martino-IST, Genova, Italy), whereas HTLA-230 cell line was kindly provided by Dr. E. Bogenmann (Children's Hospital Los Angeles, Los Angeles, CA) [Dondero et al., 2016]. NB cell lines were periodically checked for MYCN amplification by fluorescence in situ hybridization analysis. NB cell lines were cultured in the presence of RPMI 1640 medium supplemented with 10% heat inactivated FCS (Sigma-Aldrich), 50 mg/ml streptomycin, 50 mg/ml penicillin and 2 mM glutamine. and checked for morphology, proliferation rate and mycoplasma contamination, after thawing and within passages in culture.

Spike-in experiments

For spike-in experiments a different number of HTLA-230 cells ranging from 2000 to 2 cells were mixed to 2x106 cells of PBMC isolated from healthy donors in a final volume of 500 ul. The mixture of cells or the corresponding number of HTLA-230 alone were analyzed using the described MFC method and results are reported in Tab.S3.

FIGURES

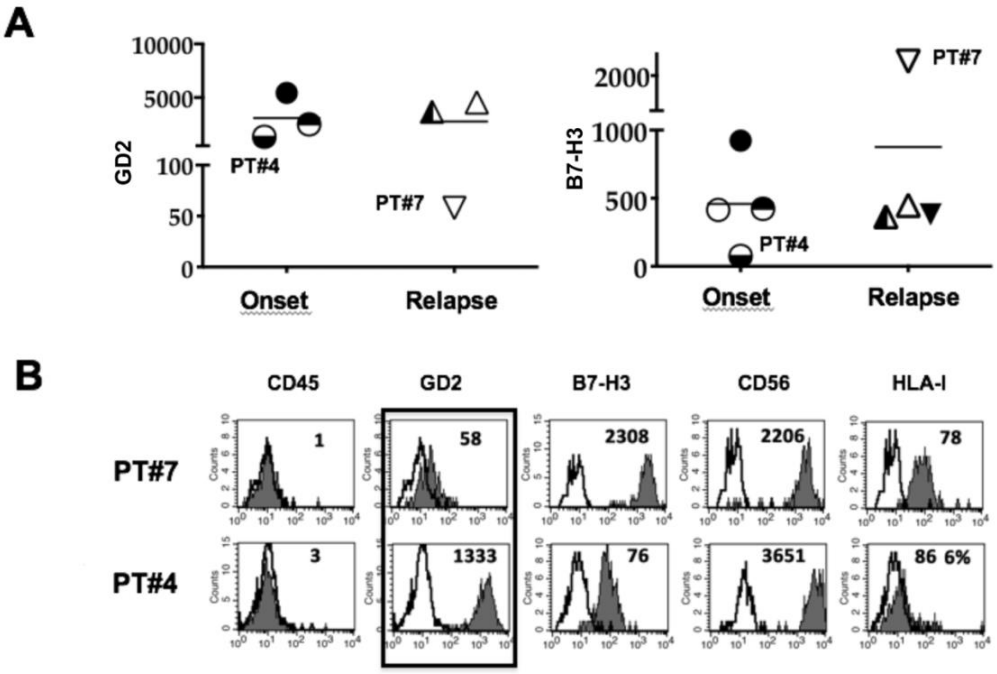

Figure S1. GD2 and B7-H3 expression in NB cell enriched bone marrow aspirates.

(A) Bone marrow aspirates from 8 NB patients (see table S1) were enriched by CD45 immunomagnetic depletion and evaluated by immunofluorescence and single-color flow cytometry for GD2 or B7-H3 expression. Mean Fluorescence Intensity (MFI) is indicated.

(B) Representative cytofluorimetric analyses (PT#7 and PT#4). White profiles refer to cells incubated with isotype-matched controls. Values inside each histogram refer to MFI.

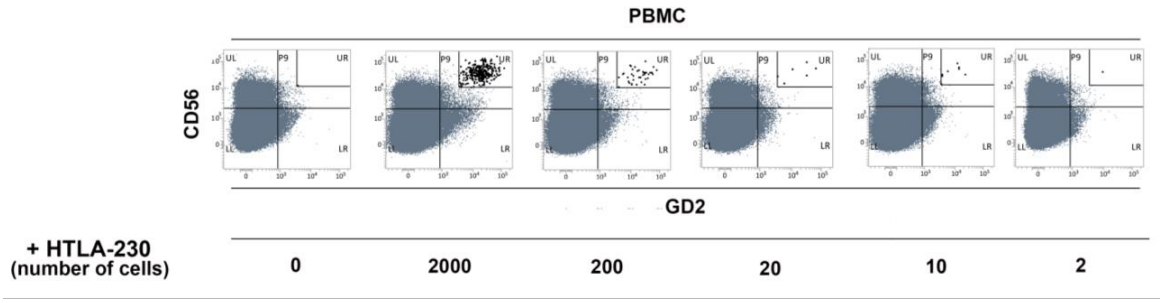

Figure S2. Spike-in experiments.

Different numbers (from 2000 to 10) of HTLA-230 cells were mixed with $2x10^6$ PBMC isolated from healthy donors and analyzed by applying our MFC method. Co-expression of CD56 and GD2 molecule in a representative experiment is shown. NB cell are highlighted in black.

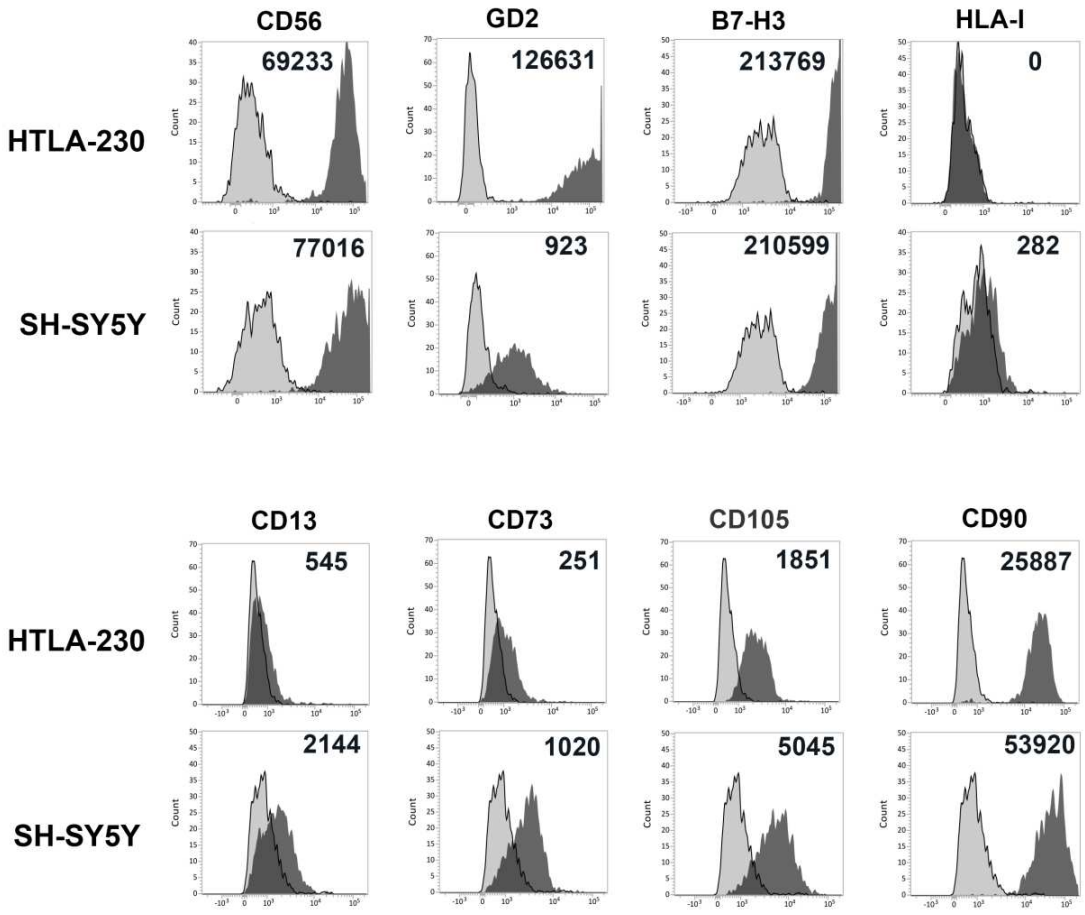

Figure S3. Expression of mesenchymal-associated markers in prototypical NB cell lines.

HTLA-230 and SH-SY5Y neuroblastoma cell lines were analyzed by immunofluorescence and single-color flow cytometry for the expression of the indicated molecules, A representative experiment is shown. White profiles refer to cells incubated with isotype-matched mAbs. Value inside each histogram refer to MFI and were calculated by subtracting MFI of isotype-matched controls.

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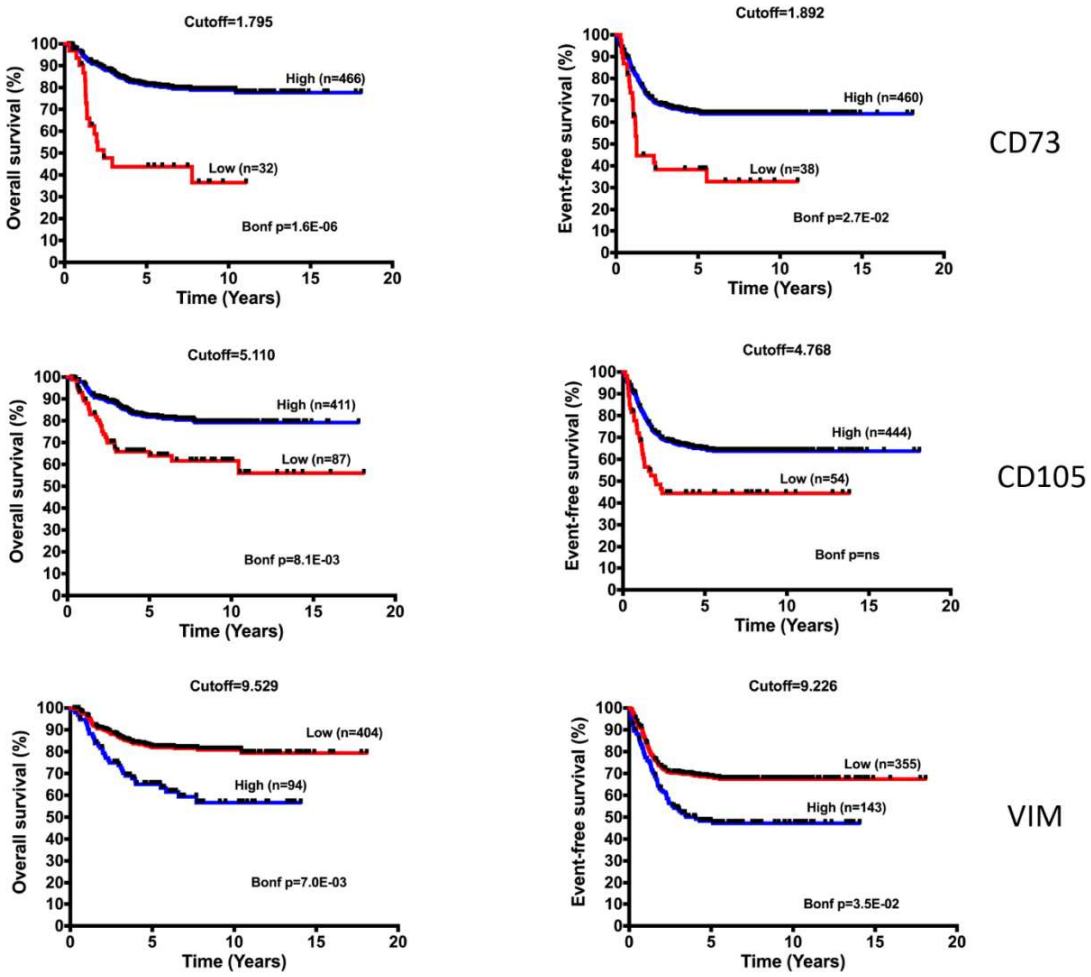

Figure S4. Kaplan-Meier curves for overall (OS) and event-free survival (EFS) of NB patients stratified by CD73, CD105 or vimentin (VIM) expression.

Kaplan-Meier plots for OS and EFS of a population of 498 NB patients (Y-axes) in a specified time interval expressed in years (X-axes). Patients were stratified according to CD73, CD105, or vimentin (VIM) gene expression. Gene symbol was reported on the right. Cut off for high or low selected marker expression was chosen by Kaplan-Meier scan method and was displayed on top of each plot. The number of patients with high or low marker expression was displayed on top of each curve. Survival curves relative to high marker expression were colored in blue. While survival curves relative to low marker expression were colored in red. The black symbol upon a survival curve indicated that a patient was

lost at follow-up time. Significance of the differences between two survival curves was assessed by log-rank test. P-values were corrected for multiple hypotheses testing by Bonferroni method and displayed in the bottom right part of each plot (Bonf p). Corrected P-values lower than 0.05 were considered statistically significant

TABLES

PATIENT	STAGE	PHASE	GD2 (MFI)	B7-H3 (MFI)
#1	M	ONSET	5425	922
#2	M	ONSET	2503	422
#3	M	ONSET	ND	414
#4	M	ONSET	1333	76
#5	M	RELAPSE	3685	365
#6	M	RELAPSE	4552	449
#7	L	RELAPSE	58	2308
#8	M	RELAPSE	ND	382

Table S1. Clinical features and Mean Fluorescence Intensity (MFI) of GD2 and B7-H3 molecules of patients PT#1-PT#8. Samples were provided by Ospedale Pediatrico Bambino Gesù and IRCCS G. Gaslini.

PATIENT ID	Age at diagnosis (months)	INRG (Stage)	Risk Group	MYCN status	Histopathological Diagnosis	Age at relapse
4904	57	M	High-Risk	Gain	NB, Schwannian stroma poor, NOS.	No relapse
4908	9	L2	Low-Risk	Not amplified	NB, Schwannian stroma poor, poorly differentiated. Low MKI, low M.I Favourable Histology according to INPC (age-linked).	No relapse
4914	7	MS	Low-Risk	Not amplified	NB, Schwannian stroma poor, NOS.	No relapse
4650	49	L2	Intermediate-Risk	Not amplified	NB, Schwannian stroma poor, NOS.	74
4929	<1	L1	Intermediate-Risk	Not amplified	NB, Schwannian stroma poor, poorly differentiated. Low MKI, low M.I. Favourable Histology according to INPC.	8
4930	26	M	High-Risk	Amplified	PNT, NOS.	No relapse
4932	2	L2	Low-Risk	Not amplified	NB, Schwannian stroma poor, poorly differentiated. Low MKI; high M.I. Favourable Histology according to INPC.	9
4814	38	M	High-Risk	ND	PNT, NOS.	53
4547	58	M	High-Risk	Gain	PNT, NOS.	76
4668	90	L2	Intermediate-Risk	Not amplified	Ganglioneuroblastoma nodular, with NB, Schwannian stroma poor, poorly differentiated component. Unfavourable Histology according to INPC.	113

4944	84	M	High-Risk	Not amplified	Diagnosi made on Bone Marrow aspirate. Histology not available.	No relapse
4938	21	L2	Intermediate-Risk	Not amplified	NB, Schwannian stroma poor, NOS.	No relapse
4937	195	M	High-Risk	Gain	NB, Schwannian stroma poor, NOS.	No relapse
4790	1	L1	Low-Risk	Not amplified	NB, Schwannian stroma poor, NOS.	No relapse
4210	39	M	High-Risk	ND	PNT, NOS.	66
4945	40	M	High-Risk	Amplified	PNT, NOS.	No relapse
4946	24		-	-	KidneyTumor. No PNT.	No relapse
4803	8	MS	Low-Risk	Not amplified	NB, Schwannian stroma poor, NOS.	22
4950	120	M	High-Risk	Not amplified	NB, Schwannian stroma poor, NOS.	No relapse
4978	90	M	High-Risk	Not amplified	NB, Schwannian stroma poor, NOS.	No relapse
5013	36	NA	NA	Not amplified	NA	No relapse
5018	54	NA	NA	NA	N	No relapse

Table S2. Available clinical and histological characteristics of patients cohort analyzed

Samples were provided by Biobanca Integrata Tessuto-genomica–BIT (IRCCS G. Gaslini). NB=Neuroblastoma; PNT=Peripheral Neuroblastic Tumor; NOS =Not Otherwise Specified; MKI:Low (<2%); Intermediate (>2<4%), high (>4%); M.I. (mitotic index): low (<10x10 HPF), high (>10x10 HPF). HPF= high power field (40x); Ganglioneuroblastoma nodular = composite tumor (NB stroma poor component + NB stroma rich/dominant component)

		EXP1		EXP2	
HTLA-230 alone or mixed to PBMC	**HTLA-230 dilution**	**HTLA-230 alone**	**HTLA-230 mixed to PBMC**	**HTLA-230 alone**	**HTLA-230 mixed to PBMC**
2000	10^{-3}	565	495	398	319
200	10^{-4}	119	60	77	38
20	10^{-5}	9	5	10	8
10	$5x10^{-6}$	5	6	6	6
2	10^{-6}	2	0	0	0

Table S3. Spike-in experiments for the definition of the MFC sensitivity

Table summarizes data from two representative experiments. Values in the columns represent numbers of NB cells identified by MFC, alone or mixed with PBMC from 2 healthy donors.