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Oncotargets GD2 and GD3 are highly expressed in sarcomas of children, adolescents and young adults

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Abstract

Background—GD2 and GD3 are the tumor-associated glycolipid antigens found in a broad spectrum of human cancers. GD2-specific antibody is currently a standard of care for high risk neuroblastoma therapy. In this study, the pattern of GD2 and GD3 expression among pediatric/AYA (adolescent or young adult) tumors was determined, providing companion diagnostics for targeted therapy.

Methods—Ninety-two specimens of human osteosarcoma (OS), rhabdomyosarcoma (RMS), Ewing family of tumors (EFT), desmoplastic small round cell tumor (DSRCT) and melanoma were analyzed for GD2/GD3 expression by immunohistochemistry. Murine monoclonal antibody 3F8 was used for GD2 staining, and R24 for GD3. Staining was scored according to both intensity and percentage of positive tumor cells from 0 to 4.

Results—Both gangliosides were highly prevalent in OS and melanoma. Among other tumors, GD3 expression was higher than GD2 expression. Most OS samples demonstrated strong staining for GD2 and GD3, whereas expression for other tumors was highly variable. Mean intensity of GD2 expression was significantly more heterogeneous ($p < 0.001$) when compared with GD3 across tumor types. When assessing the difference between GD2 and GD3 expression in all tumor types combined, GD3 expression had a significantly higher score ($p = 0.049$). When analyzed within each cancer, GD3 expression was significantly higher only in DSRCT ($p = 0.002$). There was no statistical difference in either GD2 or GD3 expression between primary and recurrent sarcomas.

Conclusion—GD2/GD3 expression among pediatric solid tumors is common, albeit with variable level of expression. Especially for sarcoma patients, these gangliosides can be potential targets for antibody based therapies.

Keywords

GD2; GD3; melanoma; DSRCT; sarcoma

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INTRODUCTION

Cancer immunotherapy is a rapidly evolving therapeutic modality. Targeting of tumor associated antigens (TAA) holds promise of selective eradication of malignant cells. The efficacy of immune effectors, including monoclonal antibodies (mAbs) and genetically modified T-cells has been proven in clinical trials. Identifying the “ideal” target that allows for maximal selectivity and safety profile remains a challenge. Various TAAs are currently being tested by investigators for their suitability for cancer immunotherapy. Refractory and recurrent solid tumors in children and in adolescent/young adults (AYA) are particularly challenging given the lack of resources or economic incentive for target discovery, and the small number of patients available for validating safety and efficacy in clinical trials which are confounded by toxicity burdens from prior treatment-related complications. Repurposing already proven targets may offer new opportunities for recalcitrant solid tumors in children and AYA.

GD2, a disialoganglioside, is an oncofetal antigen. It is expressed on neural stem cells [1], mesenchymal stem cells [2], and breast cancer stem cells [3,4]. Postnatally its expression in normal tissues is restricted to peripheral neurons, central nervous system and skin melanocytes [5]. It probably plays a role in mediating the attachment of tumor cells to extracellular matrix proteins [6], and directs cell death signaling [7], but much is still unknown about its function in oncogenesis. GD2 has a high level of expression in neuroblastoma [8]. Other malignant tumors such as melanoma [9], soft tissue sarcomas [10], Ewing sarcoma [11], osteosarcoma [12], desmoplastic small round cell tumor (DSRCT)[13] also express GD2. GD3, a ganglioside further upstream in the biosynthesis of GD2 [14], is expressed in the majority of human melanoma tissues and melanoma cell lines, although normal melanocytes can also express this antigen [15]. GD3 expression is associated with proliferation, adhesion and invasive activity of melanoma cells [16,17] and it enhances (along with GD2) malignant properties of osteosarcoma [18]. GD3 is also present on breast cancer stem cells [4]. GD2 is a proven target for monoclonal antibodies in high risk metastatic neuroblastoma [19,20]. GD3 has also been tested extensively with promising results as an antibody target in human cancers [21-25], although no randomized efficacy trials have yet been performed. In this study, we evaluated the expression of GD2 and GD3 in a large panel of pediatric/AYA tumors of different diagnoses to assess their potential as targets for antibody based therapies.

MATERIALS AND METHODS

Tumors

Ninety two samples of human sarcomas, melanoma and DSRCT were analyzed. All tumors were snap-frozen in Tissue-Tek OCT (Miles Laboratories, Inc., Elkhart, IN) with liquid nitrogen and stored at -80°C . They were studied for GD2 and GD3 expression by immunohistochemical staining using the avidin-biotin complex (ABC) immunoperoxidase method. Clinical information was retrieved with approval of institutional review board.

Immunohistochemical Studies

Mouse IgG3 mAb 3F8 and R24 were purified as previously described [26]. Tissue sections were prepared at 7 μ m thickness. The presence of tumor cells in each section was verified by hematoxylin and eosin staining. ABC immunoperoxidase staining was initiated by fixing the tissues in cold acetone for 30 minutes at -20°C . After rinsing in phosphate-buffered saline (PBS), slides were immersed in 0.1% hydrogen peroxide for 15 min at room temperature (RT). Avidin and biotin solutions (VECTOR Laboratories, Burlingame, CA) were added sequentially for 30 min each at RT. To block non-specific binding the slides were incubated in 10% horse serum in PBS for 1 hour at RT. Each subsequent step was followed by washing of slides with PBS. Two hundred μ l of mouse IgG3-3F8 (GD2 staining) or R24 (GD3 staining), was added to the tissue sections at a concentration 1.0 μ g/ml for 1 hour. Then, 200 μ l of biotinylated horse anti-mouse IgG (H+L) antibody at 1:500 dilution in 1% horse serum was added for 30 minutes at RT followed by applying 100 μ l of ABC (VECTOR Laboratories, CA) for 30 min at RT. Thereafter, 200 μ l of DAB peroxidase substrate (VECTOR) was added to each tissue section for 2 min followed by rinsing with running tap water for 5 min. All the above reactions were performed at RT in a humidified chamber. Counterstaining was performed with Myer's hematoxylin. The slides were dehydrated by dipping sequentially in 75%, 95% and 100% ethanol, and finally in xylene. After wiping off xylene, slides were mounted with 1 drop of Cytoseal (Richard-Allan Scientific, Kalamazoo, MI) for examination. The results of staining were recorded according to the intensity of the color reaction. The percentage of tumor cells stained was calculated. Reference standards included control sections of GD2+ and GD3+ melanomas, neuroblastoma and GD2/GD3 negative tissue (muscle). Irrelevant mouse IgG3 FLOPC 21 at 1 μ g/ml was also included. Stained slides were analyzed using a Nikon Inverted Microscope ECLIPSE (Nikon Instruments Inc, Japan) attached to a CCD (Diagnostic Instruments, Sterling Heights, MI).

Staining intensity scoring

The tissue staining intensity of the samples was compared with positive and negative controls. All slides were scored from 0 to 4 according to 2 components: staining intensity and percentage of positive cells (see Supplemental Table I). Tissue was assessed and graded by 2 independent observers.

Statistical Analysis

Kruskal-Wallis test and Wilcoxon rank sum test were used for analysis of the mean difference between tumor types and between values of GD2 and GD3 (using paired differences). A P value of <0.05 was considered to be statistically significant.

RESULTS

Demographics

Available tumor samples from 92 patients with different cancer types were the subjects of this study. Sixty eight samples were obtained at diagnosis and 20 samples at the time of recurrence. Four patients were lost to follow up. Distribution of cancer diagnoses is shown in

table I. Mean patient age was 15.5 years for OS, 17.8 years for EFT, 10.4 years for RMS, and 19.1 years for DSRCT. Samples from patients with melanoma were used as a reference.

GD2/GD3 expression in tumors

Ganglioside expression by immunohistochemistry among these tumors is tabulated in table II. Both antigens GD2 and GD3 were highly expressed in OS and melanoma. In other cancers GD2 expression was positive in 50% of tumors. In contrast, GD3 had a high prevalence across all tumor types. Ganglioside expression intensity was scored as described in Methods. Most OS samples demonstrated uniformly strong staining for GD2 and GD3, whereas the intensity of expression for other tumors was highly variable (table III). Mean intensity of GD2 was more heterogeneous across tumor types ($p < 0.001$) (table IV). It was highest in OS, followed by melanoma and RMS. GD3 was not significantly different across tumor types ($p = 0.57$). When assessing the difference between GD2 and GD3 expression in all cancers combined, GD3 expression had significantly higher score ($p = 0.049$). When analyzed separately within each cancer diagnosis, GD3 expression intensity was significantly higher only in DSRCT (0.002), and it did not differ in the other tumor types.

GD2/GD3 expression intensity in primary and recurrent tumors

Samples from recurrent tumors were available for OS, EFT and RMS. For all three tumor types there was no statistically significant difference in either GD2 or GD3 expression between primary and recurrent tumors (table V).

DISCUSSION

Solid tumors represent about 50% of all malignancies in children/AYA. Despite improvements in long term survival, there remains a substantial proportion of tumors that are still recalcitrant to current therapies. Identifying actionable targets on tumor cells is a prerequisite for the development or adoption of selective therapy. RMS, OS and EFT are the most common sarcomas in children and AYA. Over the past decades survival has improved for local-regional tumors, but the cure rate for metastatic or recurrent forms has stagnated. The search for novel therapeutics with improved efficacy and favorable toxicity profile is of a paramount importance. Since GD2 and GD3 expression is mostly restricted to malignant cells, and given the safety clinical profile so far, these antigens are attractive targets for immunotherapeutics.

In this retrospective analysis of 92 tumor samples, both GD2 and GD3 were highly prevalent among OS and melanoma. Among RMS, EFT and DSRCT, while GD3 was highly prevalent (70%), GD2 expression did not exceed 50%. Most OS samples demonstrated uniformly strong staining for GD2 and GD3, whereas the intensity of expression for other tumors was variable. Mean intensity of GD2 was highest in OS, followed by melanoma and RMS. GD3 was not significantly different across tumor types. When assessing the difference between GD2 and GD3 expression, GD3 had significantly higher intensity score. When analyzed among individual tumor types, the difference in GD3 versus GD2 expression was significant only for DSRCT. There was no significant difference in either GD2 or GD3 expression

between primary and recurrent OS, EFT and RMS, although the small sample size had limited any definitive conclusions.

The introduction of murine monoclonal antibodies (e.g. 3F8 [26] and 14G2a [27]) specific for the penta-saccharide moiety of GD2 initiated a new era of neuroblastoma immunotherapy more than two decades ago. The Children's Oncology Group (COG) landmark trial demonstrated the efficacy of anti-GD2 antibody ch14.18 used for remission consolidation, and this therapy has become the standard of care for patients with high-risk neuroblastoma [20]. The murine anti-GD2 antibody 3F8 has also achieved long term remissions with no notable late toxicities past 20 years [19]. Humanized 14.18 (hu14.18-K322A) was proven safe although HAMA was detected in 40% of patients [28]. Humanized 3F8 (hu3F8) [29] has also been proven safe and less immunogenic than its murine counterpart [30]. Pain during antibody infusion was the major acute toxicity that required sophisticated analgesics [20,30-32]; occasional patients also developed neuropathy [33]. However, with long term followup over two decades, there were no late toxicities noted with mouse 3F8 [19]. A number of clinical studies with GD3-specific antibodies have also been reported, albeit mostly in patients with adult cancers [21-23]. Seminal trials in melanoma using R24 (GD3-specific IgG3 antibody) demonstrated a major tumor regression in patients with advanced disease [22]. Most common toxicities of GD3 antibody include pruritus and urticaria [21,34]. The high prevalence of GD3 expression in sarcomas and DSRCT shown in this study should provide a rationale for future antibody strategies directed at these tumors. There was no significant difference in intensity of GD2 and GD3 expression on melanoma in our study, albeit higher GD3 expression was found by others [35]. On the other hand, with the exception of OS, GD2 expression was found to be more heterogeneous both between tumors and within tumors, which could pose an obstacle for effective tumor targeting. To overcome this antigen heterogeneity, dual targeting strategies may be an alternative. A phase I study of a bivalent GD2/GD3 vaccine in neuroblastoma has yielded promising results [36], suggesting that using more than one target was safe and might enhance the overall anti-tumor response.

Ganglioside expression intensity was comparable between GD2 and GD3 for all tumor types with the exception of DSRCT, where the mean intensity of GD3 was significantly higher. This tumor is particularly challenging with long term-survival never exceeding 15% [37]. Current multimodal (surgery, radiation and chemotherapy) therapy is dose-limiting given the severe long term treatment related toxicities [38]. Even after achieving minimal residual disease or complete clinical remission, most patients succumb to tumor recurrence. Although, DSRCT expresses an array of various neuroectodermal, mesenchymal and epithelial markers [39,40], most of these targets are not druggable because of their intracellular localization or cross-presentation on normal tissues. With the discovery of surface B7-H3 antigen on DSRCT cells [13], radioimmunotherapy using the specific antibody 8H9 (NCT01099644) has produced some long term remissions, although cure is still elusive [41,42]. The high expression of GD3 in DSRT should provide another actionable target especially if the potential of dual specificity approach against GD3 and GD2 [13] can be exploited.

In our study the expression of either ganglioside did not differ between primary and recurrent sarcomas in a relatively small size sample set. Although increased GD2 expression was reported in OS upon recurrence [43], a subsequent report by the same group did not confirm the difference between matched samples (primary and recurrent) [44]. Our observations are consistent with this report, providing additional rationale for both GD2 and GD3 directed immunotherapy, whether in first response or after recurrence for OS, RMS, EFT, and DSRCT.

The success of GD2-directed therapy in neuroblastoma has not yet been reproduced with other TAA-targeted agents and with other tumor types with an exception for herceptin specific for HER2 in breast cancer. Several attempts to exploit antibody-based therapy including anti-IGF-1R in relapsed and refractory sarcomas [45], anti-HER2 in osteosarcoma [46], anti-TRAIL-2 in pediatric sarcomas [47], and anti-VEGFR in recurrent RMS [48], have not shown substantial clinical benefits. The success of GD2-directed therapy in neuroblastoma may be because of the nature of tumor target (where the epitope is close to the cell membrane) with restricted expression in normal tissues [49], the use of immunotherapy at the time of minimal residual disease, the timing of immunotherapy soon after high dose chemotherapy to avoid or prevent neutralizing antibodies, and the addition of GM-CSF. These approaches may prove useful if GD3 and GD2 are chosen as targets for antibody-based therapies for recalcitrant solid tumors in children and AYA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABC	avidin-biotin complex
AYA	adolescent or young adult
COG	Children's Oncology Group
DSRCT	desmoplastic small round cell tumor
EFT	Ewing family of tumors
mAbs	monoclonal antibodies
OS	osteosarcoma
PBS	phosphate-buffered saline

RT	room temperature
RMS	rhabdomyosarcoma
TAA	tumor associated antigens

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Table I

Diagnoses in 92 tumor samples in this analysis

Tumor type	Number of cases	Primary/recurrence
Osteosarcoma	19	9/6 (4 patients lost to follow up)
Ewing family of tumors	15	9/6
Rhabdomyosarcoma	18	10/8
Melanoma	20	20/0
Desmoplastic small round cell tumor	20	20/0

Table II

GD2/GD3 expression by tumor type

Staining	OS		EFT		RMS*		Melanoma		DSRCT	
	GD2	GD3	GD2	GD3	GD2	GD3	GD2	GD3	GD2	GD3
negative (score of 0)	3	3	9	4	9	3	5	2	18	6
positive (score of 1, 2, 3 or 4)	16	16	6	11	9	14	15	18	2	14
% positive	84	84	40	73	50	82	75	90	10	70

* one tumor sample in this group was not assessed for GD3 expression

Table III

GD2/GD3 expression intensity score by tumor type

staining score	OS n = 19		EFT n = 15		RMS* n = 18		Melanoma n = 20		DSRCT n = 20	
	GD2	GD3	GD2	GD3	GD2	GD3	GD2	GD3	GD2	GD3
0	3	3	9	4	9	3	5	2	18	6
1	2	9	3	2	3	6	4	1	1	3
2	2	4	1	5	1	5	7	14	0	4
3	10	2	0	3	3	2	4	3	1	5
4	2	1	2	1	2	1	0	0	0	2

* one tumor sample in this group was not assessed for GD3 expression

Table IV

Mean intensity score by tumor type

Tumor type	mean intensity score		p-value
	GD2	GD3	
Osteosarcoma	2.32	1.42	0.055
Ewing family of tumors	0.87	1.67	0.13
Rhabdomyosarcoma	1.22	1.53	0.66
Melanoma	1.5	1.9	0.22
Desmoplastic small round cell tumor	0.2	1.7	0.002

Table V

Primary and recurrent tumor GD2/GD3 expression mean scores

	OS		EFT		RMS	
	GD2	GD3	GD2	GD3	GD2	GD3
primary	2.78	1.33	0.78	1.56	1.1	1
recurrent	1.67	1.33	1	1.83	1.38	2.12
p-value	0.09	0.75	0.69	0.67	0.57	0.06