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Expression of glioma-associated antigens in pediatric brain stem and non-brain stem gliomas

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Abstract

We investigated the protein expression of three glioma-associated antigens (GAAs) in pediatric brain stem glioma (BSG) and non-brain stem glioma (NBSG) cases with a view to their possible use in immunotherapy. Expression of EphA2, IL-13Rα2 and Survivin were studied by immunohistochemistry on paraffin-embedded tissues using a series of 15 BSG cases and 12 NBSG cases. Thirteen of 15 BSGs and all 12 NBSGs expressed at least one of GAAs; and 7 BSGs and 9 NBSGs expressed at least two of these GAAs at higher levels than non-neoplastic brain. There was no association between the tumor grade and levels of GAA expression. Although many cases demonstrated diffuse expression of GAAs throughout specimens, partial or patchy expression was noted in a small number of cases, suggesting a need for targeting multiple GAAs in immunotherapy. These results suggest that EphA2, IL-13Ralpha2 and Survivin are suitable targets for developing vaccine strategies for pediatric glioma.

Keywords

Brain stem; EphA2; Glioma; Glioma-associated antigen; Immunohistochemistry; interleukin-13 receptor alpha 2; Pediatric glioma; Survivin

Introduction

Children with intrinsic brainstem malignant gliomas have 1- and 5-year progression-free survival rates of less than 25% and 10%, respectively [1,2]. Radiologically, these lesions generally have a characteristic appearance on magnetic resonance imaging that, in most instances, obviates the need for a biopsy to establish the histological diagnosis in children with an appropriate clinical history [3]. Accordingly, in recent years, these lesions have seldom been biopsied. In the limited numbers of cases where tissue has been obtained, the diagnosis was most often high-grade glioma [3–7]. Given the paucity of information about the biology of brain stem gliomas, it is often necessary to make inferences about their molecular characteristics based on studies of pediatric highgrade gliomas at non-brain stem sites. Other

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than radiation therapy, no other therapy has demonstrated efficacy in prolonging the survival of these patients [8].

Similarly, pediatric patients with incompletely resected anaplastic astrocytoma or glioblastoma multiforme also have a dismal prognosis with contemporary therapy, with 1- and 5-year progression-free survival rates of less than 50% and 20%, respectively. These lesions show a low frequency of objective responses to conventional chemotherapeutic agents, which is less than 20% even for some promising agents, such as temozolomide [2].

In view of these discouraging results, there is a strong need for identifying new therapeutic approaches that target those features of the tumor cell that distinguish it from surrounding normal cells, such as vaccine strategies targeting glioma-associated antigens. In recent years, we have examined the applicability of a series of tumor cellbased immunization approaches for patients with gliomas [9–15]. Although promising results have been achieved [9,16,17], a limitation of this strategy is the need for autologous tumor and the time delay involved in generating a patient-specific vaccine. In contrast, vaccines in the form of synthetic peptides encoding T-cell epitopes in GAA would eliminate the need for autologous fresh glioma explants to generate clinical grade vaccines, facilitate timely vaccine production, and potentially reduce the risk of autoimmune encephalitis. To this end, we have focused attention on the identification and characterization of human GAA-derived cytotoxic T-lymphocyte (CTL) epitopes, such as those in the interleukin-13 receptor (IL-13R)α2 [18,19] and EphA2 [20]. Recent studies by others have shown that Survivin, which contains HLA-A2-restricted CTL epitopes [21,22], are frequently expressed at high levels in grade II, III and IV astrocytomas in adults [23]. In vivo, glioma cells may undergo “immuno-editing”, in which heterogeneous progressive lesions exhibit loss of specific antigens [24]. Hence, an optimal glioma vaccine design should include multiple GAA-derived T cell epitopes.

In the current study, we performed immunohistochemical evaluation of three GAAs, EphA2, IL-13Rα2 and Survivin in paraffin-embedded tissues derived from pediatric gliomas arising from both brain stem and non-brain stem locations. Our results suggest that EphA2, IL-13Rα2 and Survivin are suitable targets for developing vaccine strategies for pediatric glioma.

Materials and methods

Tissues

Archival formalin-fixed, paraffin-embedded glioma specimens (15 BSG, 12 NBSG), obtained at the time of tumor biopsy or resection or by autopsy, were provided for this study under an Institutional Review Board-approved protocol. The vast majority of the BSG samples were obtained prior to the MR era, and in many cases prior to the availability of high resolution CT, when open biopsy was often performed to establish the diagnosis, whereas the NBSG samples were obtained more recently. Normal brain sections were obtained from Human Brain Tissue Bank, Division of Neuropathology of the University of Pittsburgh.

Antibodies

The following primary antibodies were used at indicated dilutions or concentrations (in parentheses): anti-human EphA2 monoclonal antibody (mAb) (1:100; Ab 208, mouse IgG1, MedImmune), anti-human IL-13Rα2 polyclonal antibody (pAb) (15 μg/ml; AF146, goat polyclonal, R&D), anti-human Survivin pAb (1:100 in 1% BSA, C-19, goat polyclonal, Santa Cruz Biotechnology, Inc.) and antiglial fibrillary acidic protein (GFAP) (1:500, rabbit polyclonal, DakoCytomation). The secondary antibodies used in the current study were peroxidase-conjugated rabbit polyclonal anti-goat IgG heavy and light chains (1:200; ab6741,

Abcam), goat polyclonal anti-mouse IgG heavy and light chains (1:200; AP124P, Upstate Cell Signaling) and goat polyclonal anti-rabbit IgG heavy and light chains (1:500; AP132P, Chemicon International).

Immunohistochemistry

Paraffin-embedded tissue sections (6–8 μm) were deparaffinized in xylene and rehydrated in a series of ethanol/phosphate-buffered saline (PBS) washes. Antigenicity was retrieved with modified citrate solution (Dako) in a pressure cooker for 20 min. Endogenous peroxidase was blocked by incubation in 3.0% hydrogen peroxide in PBS, and the nonspecific binding of antibodies was blocked by incubation in serum-free blocking solution (Dako) for 1 h. The slides were then incubated with each of antigen-specific antibodies diluted in 1% bovine serum albumin (BSA) in PBS overnight at 4°C. After washing, slides were then incubated with corresponding peroxidase-conjugated secondary antibodies for 1 h at room temperature. Peroxidase labeling was visualized using Vectastain's Nova Red kit. The sections were lightly counterstained with Gill's hematoxylin. The sections were then reviewed. Positive staining (+) was defined by definite, but moderate staining in the tumor. Strong positive staining (2+) was defined by intense immunoreactivity. In both positive (+) and strong positive (2+) cases, it was also con- firmed that positive signals in the tumor tissues were higher than signals from normal tissue elements in the same sections or normal brain control sections.

Results

The expression of EphA2, IL-13Rα2 and Survivin protein was evaluated by immunohistochemistry in 15 BSG and 12 NBSG samples. Figure 1 demonstrates representative cases. A brain stem (medullary) low grade glioma (BSG-1) showed diffuse expression of IL-13Rα2 and Survivin, but was negative for EphA2.A temporo-frontal GBM (NBSG-1) and a thalamic GBM (NBSG-10) both exhibited strong expression of EphA2, and moderate IL-13Rα2 and Survivin. Interestingly, EphA2 was also expressed in tumor-associated vascular endothelial cells (NSBG-1), consistent with previous findings by us [25] and others [26].

Staining results of all evaluated cases are summarized in Table 1. Negative staining is defined by lack of staining or weak background staining that was not higher than the background signal observed in negative control samples. Although many cases demonstrated diffuse expression of GAAs throughout specimens, partial or patchy expression was noted in a small number of cases, exemplified by Survivin expression in NBSG-1 (Fig. 1).

Of our the entire series of 15 BSG and 12 NSBG samples, 7 of 15 BSG and 12 of 12 NBSG cases were positive for EphA2, 10 of 15 BSG and 7 of 9 evaluated NBSG cases exhibited positive staining for IL-13Rα2, and 8 of 15 BSG and 8 of 12 NBSG cases were positive for Survivin. There were three cases that were not evaluated for IL-13Rα2 due to the limited number of available slides. Overall, 13 of 15 BSG cases and all 12 NBSG cases expressed at least one of GAAs; and 7 of 15 BSG and 9 of 12 NBSG expressed at least two of these GAAs. Table 2 summarizes expression of the three GAAs, subdivided by tumor location and histological grade.

Our current series does not demonstrate any clear association between the tumor grade and levels of GAA expression.

Discussion

To our knowledge, this is the first report evaluating expression of three GAAs: EphA2, IL-13Rα2 and Survivin, in pediatric gliomas, including brain stem gliomas. We chose to evaluate these three antigens because they are expressed in adult gliomas and are known to

contain CTL epitopes, especially in the context of HLA-A2 [18,20,23]. Our results suggest that EphA2, IL-13α2 and Survivin are suitable targets for developing vaccine strategies for pediatric glioma.

EphA2, which was expressed in 7 of 15 BSG and all 12 NBSG cases in the current study, is a tyrosine kinase receptor that plays a role in carcinogenesis [27,28]. In normal cells, EphA2 localizes to sites of cell-to-cell contact [29,30], where it may negatively regulate cell growth. EphA2 is frequently overexpressed and often functionally dysregulated in advanced cancers, contributing to their malignant phenotype [31]. We have previously reported that $EphA2_{883–891}$ can elicit an HLA-A2-restricted CTL response against glioma cell lines [20]. In addition, recent studies from us [20] and others [32] have revealed that a majority of adult malignant gliomas express high levels of EphA2. More recently, EphA2 mRNA overexpression was found to correlate inversely with survival in a panel of 21 adult GBM cases, and ligand-mediated EphA2 receptor activation increased GBM cell proliferation via a mitogen-activated protein kinase-dependent pathway [33]. These findings suggest that targeting of EphA2 by immunotherapy may have efficacy in controlling tumor growth.

In the current study, more than a half (8 of 15) BSGs were negative for EphA2, whereas all 12 NBSG cases were positive. Although this may represent biological differences between these tumor types, it is important to emphasize that the BSG tissues evaluated in the current study were much older than the NBSGs, in some cases from specimens obtained in 1950s and 1960s, or obtained by autopsy. Thus, even though the BSG tissues generally demonstrated strong immunoreactivity for GFAP, it is possible that the antigenicity of these specimens for less robust targets may not have been as well preserved as NBSG tissues.

IL-13Rα2 is a membrane glycoprotein that mediates activation of the TGF-β1 promoter upon stimulation by IL-13 (or IL-4) and tumor necrosis factor (TNF)-α [34]. IL-13Rα2 has attracted significant attention as a target for glioma therapy [35] because this receptor is overexpressed by more than 80% of adult malignant gliomas but is not expressed at detectable levels in normal brain tissues or at high levels in other normal organs except the testis [36]. We recently found that an analogue peptide of natural IL-13R$\alpha2_{345–353}$ [19], in which the first and ninth amino-acid residues, tryptophan and isoleucine, were replaced by valine and alanine, respectively, could elicit a greater CTL response against HLA-$A2^+$, IL-13R$\alpha2^+$ glioma cells compared to the natural peptide (IL-13R$\alpha2_{345–353:1A9V}$) [37]. Kawakami et al. have previously observed with a total 58 pediatric brain tumor cases that one hundred percent (11 of 11) high-grade astrocytoma, 79% (26 of 33) low-grade astrocytoma, 67% (4 of 6) medulloblastoma, and 67% (2 of 3) ependymoma samples expressed IL-13Rα2 [38]. Taken together with our current study, in which 10 of 15 BSGs and 7 of 9 NBSGs demonstrated immunoreactivity for IL-13Rα2, it seems promising to target IL-13Rα2 in future vaccine trials for pediatric brain tumor patients.

The apoptosis inhibitor protein Survivin also appeared to be expressed at high levels in a majority of cases evaluated in the current study. Survivin is overexpressed in most human cancers, and inhibition of its function results in increased apoptosis [39]. Induction of cytotoxic immunity against Survivin may, therefore, be an attractive strategy [40]. Survivin has multiple T cell epitopes, including $Survivin_{96–104}$ as an HLA-A2-restricted CTL epitope [22,41]. Moreover, vaccination of a pancreatic cancer patient with a modified Survivin epitope peptide, $Survivin_{96–104:2M}$ in which the second residue threonine was replaced by methionine, induced complete remission of a liver metastasis [42]. Further, vaccinations using dendritic cells loaded with $Survivin_{96–104}$ induced positive interferon (IFN)-γ ELISPOT responses in four of five patients with advanced melanoma [21]. A recent immunohistochemical study demonstrated that 100% of adult astrocytoma specimens (n = 29; grades II–IV), but not normal brain tissues, contained Survivin-positive cells [23]. The mean percentage of immunoreactive cells in each specimen was 70.0 in grade II, 81.3 in grade III, and 85.0 in grade IV. Interestingly, high level

expression of Survivin was correlated with poor prognosis in patients with grade II or III astrocytomas [23]. Even though we have not been able to demonstrate a correlation between Survivin expression levels and survival of pediatric patients in the current study, these expression data support the inclusion of Survivin in vaccine strategies for pediatric patients with glioma.

In conclusion, this study demonstrates the frequent expression of three therapeutically exploitable GAAs (EphA2, IL-13Rα2 and Survivin) in archival cohorts of BSGs and hemispheric NBSGs of childhood. All but two of 15 BSG cases and all 12 NBSG cases evaluated in the current study expressed at least one of these antigens. In addition, although many cases in the current study demonstrated diffuse expression of GAAs throughout specimens, partial or patchy expression was also noted in a small number of cases, suggesting the need for targeting multiple GAAs in immunotherapy.

Given the availability of CTL epitopes for these antigens, development of novel vaccine trials targeting these GAAs for pediatric gliomas is warranted.

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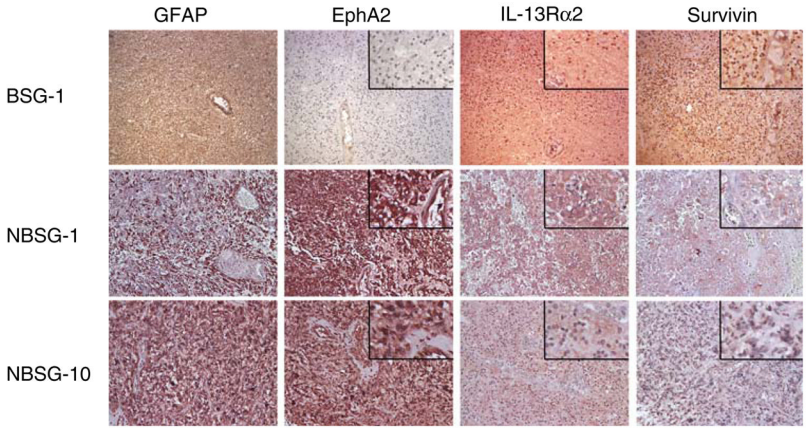

Fig. 1.

Immunohistochemical analysis of EphA2, IL-13Rα2 and Survivin in pediatric glioma specimens. Tissue specimens were stained with specific antibodies against GFAP, EphA2, IL-13Rα2, or Survivin as described in Materials and Methods. Original magnification ×200. High power insets for EphA2, IL-13Rα2, and Survivin were derived from the corresponding low power fields

Table 1

Expression of glioma-associated antigen (GAA) in pediatric brain tumor sections by immunohistochemistry

Case	Age	Gender	Diagnosis[a]	Site	GFAP	EphA2	IL-13Ra2	Survivin
BSG								
1	5	f	Low grade	Medulla	1+	—	1+	1+
2	15	m	High-grade	Pons	2+	2+	2+	—
3	7	f	High-grade	Pons	2+	—	1+	—
4	8	f	Low-grade	Medulla	1+	—	1+	1+
5	17	m	High-grade	Pons	1+	1+	1+	2+
6	6	m	High-grade	Pons	2+	—	—	—
7	4	f	High-grade	Pons	1+	1+	1+	1+
8	7.5	f	High-grade	Pons	1+	—	1+	—
9	4	f	Low-grade	Medulla	2+	1+	1+	2+
10	7	m	High-grade	Pons	2+	1+	—	—
11	N/A		Malignant	Pons	1+	—	—	2+
12	N/A		Malignant	Pons	1+	—	—	1+
13	N/A		Malignant	Pons	1+	—	—	2+
14	4.9	m	Low-grade	Medulla	1+	2+	2+	—
15	0.7	m	Low-grade	Pons	1+	2+	1+	—
NBSG								
1	1.2	f	gbm	Temporofrontal	1+	2+	1+	1+
2	1.2	f	aa	Thalamus	1+	1+	—	—
3	16.7	f	aa	Frontoparietal	1+	2+	1+	1+
4	0.8	f	Low-grade	Frontal	1+	2+	2+	2+
5	1.4	f	aa	Basal ganglia	1+	2+	N/A	1+
6	15.6	m	aa	Frontal	1+	2+	N/A	1+
7	18	f	gbm	Frontal	1+	1+	N/A	—
8	11.5	m	Low-grade	Parieto-frontal	1+	2+	1+	1+
9	9.5	m	Low-grade	Parietal	1+	1+	1+	—
10	12.9	m	gbm	Thalamus	1+	2+	1+	1+
11	6.5	m	Low-grade	Frontal	1+	2+	1+	1+
12	6.3	f	gbm	Parietofrontal	1+	1+	—	—

N/A, information not available

[a]Because of the small sizes of many of the specimens, histological diagnoses of the brainstem gliomas are broadly characterized into low-grade and high-grade lesions—no attempt was made to distinguish presumptive grade III (AA) from grade IV (GBM) tumors

Table 2

Glioma-associated antigen (GAA) expression in brain stem (BS) and non-brain stem (NBS) pediatric gliomas Diagnosis No. of patients EphA2

Diagnosis	No. of patients	EphA2 −/1+/2+	IL-13Rα2 −/+/++	Survivin −/1+/2+
BS LGG	5	2/1/2	0/4/1	2/2/1
BS HGG	10	6/3/1	5/4/1	5/2/3
NBS LGA	4	0/1/3	0/3/1	1/2/1
NBS AA	4	0/1/3	1/1/0[a]	1/3/0
NBS GBM	4	0/2/2	1/2/0[a]	2/2/0

BS, brain stem; NBS, non-brain stem; LGG, low grade glioma; HGG, high grade glioma; LGA, low grade astrocytoma; AA, anaplastic astrocytoma, GBM; glioblastoma multiforme

[a]There were one GBM and two AA cases that were not evaluated for IL-13R*α*2 due to the limited number of slides