Journal of Neuroimmunology 277 (2014) 168–175

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Therapeutic targets in subependymoma

Ling-Yuan Kong [a,1], Jun Wei [a,1], Ali S. Haider [a,1], Brandon D. Liebelt [e], Xiaoyang Ling [a], Charles A. Conrad [b], Gregory N. Fuller [c], Nicholas B. Levine [a], Waldemar Priebe [d], Raymond Sawaya [a], Amy B. Heimberger [a,*]

[a] Department of Neurosurgery, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States
[b] Department of Neuro-Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States
[c] Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States
[d] Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States
[e] Department of Neurosurgery, Houston Methodist, Houston, TX 77030, United States

ARTICLE INFO

Article history:
Received 19 September 2014
Received in revised form 22 October 2014
Accepted 23 October 2014

Keywords:
Subependymoma
Ependymoma
Noninvasive
Immunotherapy

ABSTRACT

Subependymomas are usually treated with surgical resection; however, no standard, defined alternative medical therapy is recommended for patients who are not surgical candidates, owing to a paucity of molecular, immunological, and genetic characterization. To address this, an ex vivo functional analysis of the immune microenvironment in subependymoma was conducted, a subependymoma cytokine/chemokine microarray was constructed for the evaluation of operational immune and molecular pathways, and a subependymoma cell line was derived and used to test a variety of cytotoxic agents that target operational pathways identified in subependymoma. We found that immune effectors are detectable within the microenvironment of subependymoma; however, marked immune suppression is not observed. The subependymoma tissue microarrays demonstrated tumor expression of p53, MDM2, HIF-1α, topoisomerase II-β, p-STAT3, and nucleolin, but not EGFRvIII, EphA2, IL-13RA2, CMV, CTLA-4, FoxP3, PD-1, PD-L1, EGFR, PDGF-α, PDGF-β, PDGFR-α, PDGFR-β, PTEN, IGFBP2, PI3K, MDM4, IDH1, mTOR, or Jak2. A topoisomerase inhibitor (WP744, $IC_{50} = 0.83$ μM) and a p-STAT3/HIF-1α inhibitor (WP1066, $IC_{50} = 3.15$ μM) demonstrated a growth inhibition of the subependymoma cell proliferation. Cumulatively, these data suggest that those agents that interfere with oncogenes operational in subependymoma may have clinical impact.

1. Introduction

Ependymomas are glial neoplasms that originate from ependymal cells lining the cerebral ventricles and the central canal of the spinal cord. These tumors are designated, according to the World Health Organization (WHO) classification system of central nervous system (CNS) tumors, as myxopapillary ependymoma and subependymoma (grade I), ependymoma (grade II), and anaplastic ependymoma (grade III). Subependymomas are relatively benign and slowly growing neoplasms, commonly found within the fourth ventricle (50–60% of cases), lateral ventricles, or at the cervical/cervicothoracic area of the spinal cord (Louis et al., 2007). Surgery has been the preferred form of therapy for subependymomas and can be curative in both cerebral and spinal cases. However, recurrence has been reported due to incomplete resection. Furthermore, surgery can be complicated, especially for subependymal tumors located in the fourth ventricle with a goal of gross total resection due to their proximity and often adherence to underlying brainstem and/or cranial nerves (Scheithauer, 1978; Jooma et al., 1985; Lombardi et al., 1991). Radiotherapy could be considered, but for pure subependymomas this therapeutic strategy is of questionable benefit (Lombardi et al., 1991). Because these tumors are rare, little is known regarding potential therapeutic approaches. In this study, our goal was to elucidate relevant therapeutic targets and devise noninvasive treatment strategies for patients with subependymomas. However, to pursue this approach, advances are needed in our understanding of subependymoma biology.

Immunotherapeutic approaches are potentially beneficial, with the advantage of being minimally invasive and having negligible cytotoxic effects (Ge et al., 2010). Overexpression of specific antigens in glioma cells has been previously identified as potential therapeutic targets, and vaccines have been developed to successfully target these antigens (Ge et al., 2010). Several of these proteins, such as survivin, EphA, IL-13RA, EGFRvIII, CMV antigens, and nucleolin, have been shown to be overexpressed in gliomas, including low-grade gliomas (Heimberger et al., 2005; Mitchell et al., 2007; Okada et al., 2008; Xu et al., 2012); thus they may serve as potential targets for subependymoma immunotherapy. Peptide and aptamer vaccines are available that could target these antigens (Sampson et al., 2010, 2011; Kanwar et al., 2011).

* Corresponding author at: Department of Neurosurgery, The University of Texas MD Anderson Cancer Center, Unit 422, P.O. Box 301402, Houston, TX 77230-1402, United States. Tel.: +1 713 792 2400; fax: +1 713 794 4950.
E-mail address: aheimber@mdanderson.org (A.B. Heimberger).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.jneuroim.2014.10.008
0165-5728/

Alternatively, blockade of subependymoma operational immune suppressive pathways could also be potential therapeutic targets. A variety of agents targeting tumor-mediated immunosuppressive pathways such as CTLA-4, Tregs, and PD-1 are available or under clinical investigation (Fecci et al., 2007; Hodi et al., 2010; Topalian et al., 2012; Wainwright et al., 2014). To ascertain whether immunotherapy is a suitable therapeutic approach, it will first be necessary to clarify the antigenic profile and immune suppressive mechanisms within subependymoma.

Key signaling pathways that are commonly up regulated in gliomas are also potentially viable therapeutic targets and include the pathways of the signal transducer and activator of transcription 3 (STAT3) (Abou-Ghazal et al., 2008), the epidermal growth factor (EGF) (Heimberger et al., 2005), and the platelet-derived growth factor (PDGF) (Hermanson et al., 1992). The STAT3 pathway is a key molecular hub of tumor-mediated immune suppression, including cancer stem cell-mediated immune suppression (Wei et al., 2010), is commonly associated with gliomas, drives the malignant phenotype (Doucette et al., 2012), and is responsible for the proneural to mesenchymal glioblastoma subtype shift (Carro et al., 2010). These pathways have been previously targeted in gliomas with a variety of agents and approaches; however, their use for treatment of subependymoma ultimately requires the determination of relevant operational pathways. To that end, we have used tissue microarrays, ex vivo analysis, and in vitro cytotoxic assays to select and prioritize potential therapeutic approaches.

2. Materials and methods

2.1. Tissue microarray

Under The University of Texas M.D. Anderson Cancer Center (M.D. Anderson) IRB-approved protocol PA11-1166, a glioma tissue microarray (TMA) was constructed consisting of 24 ependymoma samples, 10 subependymoma samples, and 2 control spleen tissues from the M.D. Anderson Institutional Tissue Bank. Tissue cores were selected after inspection of hematoxylin and eosin (H & E)-stained sections from the original tissue block embedded in paraffin. The TMA construction procedure has been described previously (Wang et al., 2002; Wei et al., 2011) and included duplicated tissue cores 1.0 mm in diameter. The array was assembled using a manual Beecher arrayer (Beecher Instruments, Chicago, Illinois). The cores were duplicated for each submitted tumor. During immunohistochemistry processing, cores are occasionally lost or cannot be interpreted. When duplicate, interpretable cores were not available, the tumor was eliminated from the analysis. The final numbers of TMA samples available for analysis were 16 ependymoma samples and 7 subependymoma samples.

2.2. Antibodies

The following primary human antibodies were used at the indicated dilutions/concentrations: EGFRvIII (dilution: 1: 500; Clone: L8A4, a gift from Darell D. Bigner at Duke University), EGFR (dilution: 1:50; clone: 31G7; Invitrogen, Camarillo, CA), EphA2 (dilution: 1:250; clone: C-20; Santa Cruz Biotechnology, Santa Cruz, CA), IL-13RA2 (dilution: 15 μg/ml; R&D Systems, Minneapolis, MN), survivin (dilution: 1:100), nucleolin (dilution: 1:2000; clone: 4E2; Abcam, Cambridge, MA), CMV (dilution 1:50; clone CCH2 + DD69; DAKO, Carpenteria, CA), CTLA-4 (dilution: 1:250, Bioss, Woburn, MA), FoxP3 (dilution: 1:50; BioLegend, San Diego, CA), PD-1 (dilution: 1:100; clone: MRQ-22; Cell Marque, Rocklin, CA), PD-L1(dilution: 1:50; clone: 29E.2A3; BioLegend), PDGF-α (dilution: 1:50; clone: N20; Santa Cruz Biotechnology), PDGF-β (dilution: 1:10; clone: H-55; Santa Cruz Biotechnology), PDGFR-α (dilution: 1:50; clone: C20; Santa Cruz Biotechnology), PDGFR-β (dilution: 1:50; clone: P20; Santa Cruz Biotechnology), p-STAT3 (dilution: 1:100; clone: D3A7; Cell Signaling, Danvers, MA), HIF-1α (dilution: 1:50; Novus Biologicals, Littleton, CO), topoisomerase II-α (topo II-α, dilution: 1:50; clone: 3F6; Santa Cruz Biotechnology), topoisomerase II-β (topo II-β, dilution: 1:10; clone: H-8; Santa Cruz Biotechnology), PTEN (dilution: 1:100; clone: 6H2.1; DAKO), IGFBP2 (dilution: 1:50; clone:C-18; Santa Cruz Biotechnology), PI3K (dilution: 1:200; clone: EP380Y; Millipore, Temecula, CA), p53 (dilution: 1:100; clone:D0-7; DAKO), MDM2 (dilution: 1:100; clone: 1B10; Leica/ Novocastra, Buffalo Grove, IL), MDM4 (dilution: 1:500; clone: 2D10F4; LifeSpan BioSciences, Seattle, WA), IDH-1 (dilution: 1:250; clone: R132H; Dianova, Hamburg, Germany), mTOR (dilution: 1:100; clone EPR426(2); Epitomics, Burlingame, CA), and Jak2 (dilution: 1:75; clone: Ab-221; Abcam).

2.3. Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of the ependymoma/subependymoma TMA were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide/methanol for 10 min at room temperature before beginning antigen retrieval, which consisted of immersing the sections in a citrate-buffered solution (pH 6.0) and heating the sections in a microwave for 20 min. The sections were then cooled to room temperature for 40 min. After blocking with a protein block serum-free solution (DAKO, Carpinteria, CA), diluted antibody specific for each marker was added to the tissue arrays and incubated at room temperature. Secondary biotin-labeled antibody staining (biotinylated link universal solution; DAKO) was conducted for 60 min at room temperature. Finally, streptavidin-horseradish peroxidase (DAKO) was added to the slides and incubated for 30 min at room temperature. Color development was stopped by gently dipping the slides in distilled water. The nuclei were then counterstained with hematoxylin. The entire core was inspected under 200× magnification and the positive stained cells were categorized into 0%, less than 25%, 25% to 50%, 50% to 75%, and 75% to 100% expression quartiles. The data from the duplicated cores was then averaged for the final assigned quartile.

2.4. Subependymoma cell line derivation

The subependymoma cell line was derived from a surgically resected fourth ventricular tumor. The tissue sample was mechanically dissociated into a single cell suspension, and the immune properties of the population were analyzed. Single cells from the patient's tumor were cultured in vitro using four different growth media: neural stem cell medium (DMEM-F-12 medium containing 20 ng/ml of EGF, basic fibroblast growth factor [Sigma, St. Louis, MO], and B27 [1:50; Invitrogen, Carlsbad, CA]), CellGenix™ dendritic cell medium (CellGenix, Germany), RPMI1640 medium, and MEM-alpha medium. However, of these media, only the MEM-alpha medium was able to support sustained growth of subependymoma cells. From this in vitro culture, 50,000 cells from the 3rd and 4th passages were subcutaneously injected into nude mice ($n = 3$ and 5, respectively) but failed to grow. The glioblastoma cell line U87-MG was grown as a monolayer in DMEM medium with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air. The U87-MG glioma cell line was obtained from the ATCC (Manassas, Virginia). The subependymoma cell line was no longer viable after its 10th passage.

2.5. Ex vivo analysis of immune cell infiltration in human subependymoma

Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (RPA-T4), allophycocyanin (APC)-conjugated anti-CD8 (RPA-T8), APC-conjugated anti-CD45 (2D1), and phycoerythrin (PE)-conjugated anti-CD11b (ICRF44) antibodies were used for cell surface staining. The surface-stained cells were further subjected to intracellular staining with PE-conjugated monoclonal antibodies to human FoxP3 (clone PCH101, eBiosciences), p-STAT3 (PE-conjugated mouse anti-STAT3 antibody,