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Expression of Hedgehog ligand and signal transduction components in mutually distinct isocitrate dehydrogenase mutant glioma cells supports a role for paracrine signaling

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Abstract

Hedgehog signaling regulates the growth of malignant gliomas by a ligand dependent mechanism. The cellular source of Hedgehog ligand and mode of signaling have not been clearly defined due to the lack of methods to definitively identify neoplastic cells in glioma specimens. Using an antibody specific for mutant isocitrate dehydrogenase protein expression to identify glioma cells, we demonstrate that Sonic hedgehog ligand and the pathway components Patched1 and GLI1 are expressed in neoplastic cells. Further, Sonic hedgehog ligand production and GLI1 transcription factor expression occur in mutually distinct populations of neoplastic cells. These findings support a role for paracrine Hedgehog signaling in malignant gliomas.

Introduction

Hedgehog (Hh) signaling regulates cell growth and differentiation during embryogenesis, tissue homeostasis and tumorigenesis [1]. Cellular responses to Hh signaling are regulated by two transmembrane proteins, Patched1 (PTCH1) and Smoothened (SMO). PTCH1 suppresses the activity of SMOH and Hh ligand binding to PTCH1 inhibits this function, leading to SMOH activation of a transcriptional response through the GLI family of transcription factors. PTCH1 and GLI1 are transcriptional targets of Hh signaling, and thus their expression can be used to monitor pathway activation [2].

Mutations in Hh signal transduction components that confer ligand independent activation of the pathway have been associated with the development and growth of medulloblastoma and

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basal cell carcinoma [2]. In contrast, ligand-dependent activation of the Hh pathway has been identified in a broader array of malignancies including colon cancer, pancreatic carcinoma, lung cancer and malignant glioma [3–12]. In patient specimens from these tumor types, the Hh pathway appears to be activated in a relatively small population of cells as reflected by the expression patterns of PTCH1 and GLI1.

In tumors in which the Hh pathway is activated by a ligand-dependent mechanism, the cell types that generate and respond to Hh proteins are still a topic of debate due to conflicting data and perhaps to differences between specific tumor types. In epithelial cancers, for example, early reports indicated that neoplastic cells in digestive tract tumors and lung tumors responded to and required Hh signaling for growth [5, 13]. A subsequent study, however, reported that neoplastic epithelial cells, including those from lung cancer, do not respond to Hh signal but rather generate Hh ligand and signal to non-neoplastic stromal microenvironment [3]. More recently, two studies have reported that lung cancer cells respond to and require Hh signaling with one group reporting an autocrine mode of Hh signaling and the other concluding that further study was required to determine the mode of Hh signaling [7, 8]. Thus relevant cellular targets and modes of Hh signaling in epithelial cancers remain under investigation with an important need for clarification to optimize the clinical use of Hh pathway inhibitors [14].

In malignant gliomas, a concordance of evidence suggests that neoplastic cells respond to Hh signaling. Several potential cellular sources of Sonic hedgehog (SHH) ligand have been proposed, however, including neurons, vascular endothelial cells and neoplastic cells [9–12]. Malignant gliomas are highly invasive, and patient glioma specimens contain neoplastic cells infiltrated among non-neoplastic neuronal, glial and endothelial cells. Thus, difficulty identifying neoplastic among non-neoplastic cells in glioma specimens has contributed to uncertainty concerning the source of Hh ligand and mode of Hh signaling in gliomas.

Somatic heterozygous mutations in the enzyme isocitrate dehydrogenase (IDH) have been identified in several human malignancies, including glioma and acute myeloid leukemia [15–18]. The mutations occur at the substrate-recognition site of the enzyme and confer the gain of a novel function to utilize alpha ketoglutarate (α -KG) as substrate to produce high levels of (R)-2 hydroxyglutarate (2HG) [19, 20]. 2HG is a competitive inhibitor of multiple α -KG dependent enzymes including dioxygenases that regulate histone and DNA methylation [21].

In adult gliomas, mutations in the IDH1 and IDH2 genes occur in more than 70% of diffuse astrocytomas, oligodendrogliomas, oligoastrocytomas and secondary glioblastomas, and less than 7% of primary glioblastomas [15, 17, 22, 23]. IDH mutation is thought to occur early in the course of gliomagenesis, in a cell type that can give rise to either astrocytic or oligodendroglial tumors [23, 24] and prior to the acquisition of other genetic alterations such as 1p/19q codeletion and mutations in CIC and FUBP1 in oligodendrogliomas and mutations in TP53 and ATRX in astrocytomas [25–27]. IDH mutation associates with better prognosis among patients with WHO grades II through IV gliomas [17, 23, 28, 29] and with benefit from alkylating chemotherapy agents in patients with oligodendroglial tumors [30].

The majority of IDH mutations in gliomas lead to the substitution of histidine (H) at R132 of IDH1 [22]. As such, sequence analysis for IDH mutations and immunohistochemistry with an IDH1 antibody specific for R132H mutant protein [31] are specific and sensitive assays that are gaining routine use in adult glioma histopathology and prognostication [21, 22, 32]. Notably, the Hh pathway is operational and activated in the same glioma subtypes that commonly harbor IDH mutations [33]. Therefore, we analyzed IDH1 R132H staining in conjunction with SHH, PTCH1 and GLI1 expression in glioma specimens. Here we demonstrate that neoplastic glioma cells have the capacity to generate and respond to SHH ligand and present evidence for paracrine Hh signaling among discrete populations of glioma cells.

Materials and Methods

Tissue Procurement and processing

Glioma specimens from adult patients treated at Vanderbilt Medical Center were obtained in accordance with Institutional Review Board approval, and phenotyped and graded according to criteria established by the World Health Organization. Total RNA was extracted from one portion of each specimen to generate cDNA libraries as previously described [10, 12, 33]. Another portion of each specimen was formalin-fixed and paraffin-embedded (FFPE) for analysis of Hh ligand and pathway component expression.

IDH1 Sequencing

IDH sequencing was performed as previously described [33].

Immunofluorescence—FFPE glioma sections were deparaffinized in xylene, rehydrated in an ethanol series, and heated in 10 mM Citrate buffer pH 6.0 to retrieve masked antigens. Slides were stained with goat anti-Shh (1:100; N19, Santa Cruz Biotechnology) and mouse anti-IDH1 R132H (1:20; Dianova) antibodies as previously described [34]. For peptide blocking experiments, anti-Shh antibody was pre-incubated with Shh peptide (1:10; Santa Cruz Biotechnology) for two hours prior to tissue staining. Primary antibody staining was detected with anti goat Alexa Fluor 555 (1:500; Life Technologies) and anti-Mouse Alexa Fluor 488 (1:500; Life Technologies). Nuclei were stained with Hoescht 33342 (40ug/mL of PBS, Life Technologies) and slides were coverslipped with Prolong Gold Mounting Medium (Life Technologies). For quantification of the percentage of IDH1 R132H mutant cells that stained for Shh, 50 to 2000 cells were scored in two to ten high-powered fields per specimen.

For PTCH1, SHH and IDH1 triple and double labeling studies, FFPE glioma sections were processed as above for antigen retrieval and/or stained with goat anti-Ptch (C-terminus) (1:250; Everest Biotech), rabbit anti-SHH (1:400; Abcam) and mouse anti-IDH1 R132H (1:100; Dianova). Primary antibody staining was detected with donkey anti-goat Alexa Fluor 488 (1:400; Life Technologies), donkey anti-rabbit Alexa Fluor 488 (1:400; Life Technologies), donkey anti mouse Alexa Fluor 555 (1:400; Life Technologies), donkey anti-goat Alexa Fluor 555 (1:400; Life Technologies), or donkey anti-mouse Alexa Fluor 647 (1:100; Life Technologies). Autofluorescence was quenched by 20 minute incubation with

0.1% Sudan Black B in 70% Ethanol. Slides were mounted with Prolong Gold Mounting Medium with Dapi (Life Technologies).

Immunohistochemistry—FFPE glioma sections were processed for antigen retrieval and stained with goat anti-Shh (1:100; N19, Santa Cruz Biotechnology), goat anti-Gli1 (1:100; C-18, Santa Cruz Biotechnology), mouse anti-Gli1 (1:200; L42B10, Cell Signaling Technology), goat anti-PTCH1 (1:100; Everest Biotech), or mouse anti-IDH1 R132H (1:50) as previously described [10, 12, 33]. Immunoenzymatic detection for goat antibodies was achieved with the ImmPRESS anti-goat Ig horseradish peroxidase (HRP)-conjugated polymer detection kit (Vector Labs) and for mouse antibodies with the HiDef HRP Polymer System (Cell Marque), with 3, 3'-diaminobenzidine (DAB) as the chromogen. For Gli1/IDH1 R132H double labeling, sections were first stained with goat anti-Gli1 antibody and processed for immunohistochemical visualization and then with mouse anti-IDH1 R132H antibody and processed for immunofluorescence visualization as described above. For Gli1/Shh double-labeling, mouse anti-Gli1 staining was visualized with biotin-conjugated anti-mouse antibody (1: 500; Vector Labs) and alkaline phosphatase (AP)-conjugated streptavidin (1:500, Jackson Labs) with 5-bromo 4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) as chromogen and goat anti-Shh staining was visualized with the HiDef HRP Polymer System using DAB as chromogen.

In situ hybridization

Probes for GLI1 and SHH transcripts and reagents were obtained from Advanced Cell Diagnostics and chromogenic assays were performed according to the manufacturer's instructions.

Results

SHH expression in IDH1 mutant glioma cells

To determine whether mutant IDH1 protein staining could be used to identify neoplastic cells in glioma specimens in order to characterize Hh component expression, double immunofluorescence labeling studies were performed with antibodies against IDH1 R132H and SHH. The detection of mutant IDH1 R132H protein has previously been validated for immunohistochemical but not immunofluorescence staining [31]. Comparison of the two detection methods in formalin-fixed paraffin glioma sections verified that mutant IDH1 protein expression could be reliably detected by immunofluorescence staining (Figure S1). The specificity of SHH immunofluorescence was confirmed by staining with or without pre-incubation with a SHH-blocking peptide (Figure S2). After validating the immunofluorescence staining methods, 12 IDH1 R132H mutant gliomas were surveyed for SHH and IDH1 R132H protein expression. SHH staining was detected in a subset of IDH1 R132H-positive cells in 11 of 12 glioma specimens (Figure S3). Among the specimens the proportion of neoplastic cells that expressed SHH protein was $29.4 \pm 8.6\%$ (mean $\pm$ SEM) with a high degree of intertumor variability that did not correlate with glioma grade or histology. Intratumor variability of the proportion of neoplastic cells expressing SHH protein was also observed. The high degrees of inter- and intratumoral variability in SHH staining may reflect intratumoral cellular or genetic heterogeneity that is characteristic of

gliomas [35–38] and other malignancies [39, 40] or differences in the quality of archived FFPE blocks.

SHH and PTCH1 expression in distinct IDH1 mutant glioma cells

To assay for the expression of SHH ligand and its receptor PTCH1 in neoplastic cells, double immunofluorescence labeling was performed with IDH1 R132H. For better spatial resolution of neoplastic cells, a panel of WHO grade II tumors was selected to represent oligodendrogliomas and astrocytomas and contain lower cellularity than their anaplastic counterparts (Table 1). By this method, SHH (Figure 1A–D) and PTCH1 (Figure 1E–H) expression could be detected in neoplastic cells. The proportions of IDH1 R132H-positive cells within each glioma specimen that expressed SHH (0.5 to 16.1%) or PTCH1 (9.2 to 19.4%) were low and in most instances SHH and PTCH1 expression was largely confined to IDH1 mutant cells (Table 1). The detection of SHH or PTCH1 expression in cells scored as R132H-negative may indicate the presence of Hh pathway components in non-neoplastic cells or represent variability in IDH1 R132H staining intensities for technical or physiologic reasons. Thus, however, non-neoplastic cells may not be definitively identified by this method.

To determine whether SHH ligand and PTCH1 receptor were expressed in the same or separate subpopulations of neoplastic cells, each specimen was immunostained with SHH and PTCH1 antibodies (Figure 1I–L) and scored for the presence or absence of SHH expression in PTCH1-positive cells. The majority of PTCH1-positive cells were SHH-negative as double labeling was detected in only 0–13% of PTCH1-positive cells (Table 1). As PTCH1 is a transcriptional target of Hh signaling, SHH expression in the PTCH1-positive cells would be required for an autocrine mode of Hh signaling in gliomas. Thus, the absence of SHH staining in the majority of PTCH1-positive cells provides strong evidence that autocrine signaling is not the predominant mode of Hh signaling in gliomas. In support of a paracrine mode of Hh signaling, distinct SHH-expressing cells and neighboring PTCH1-expressing cells could be identified (Figure 1I–L) and confirmed to be in neighboring neoplastic cells by triple immunofluorescence staining with SHH, PTCH1 and IDH1 R132H antibodies (Figure 1M–P). Taken together, these data indicate that SHH and its transcriptional target PTCH1 are expressed in distinct, or non-overlapping, subpopulations of neoplastic cells in IDH1 mutant gliomas.

GLI1 expression in IDH1 mutant glioma cells

GLI1 expression was analyzed in conjunction with IDH1 R132H staining to assay for the expression of another Hh signal transduction component and transcriptional target in neoplastic cells. Faint GLI1 staining could be detected in formalin-fixed paraffin glioma sections by immunohistochemical but not by immunofluorescence staining (Supplementary Figure 4A and 4B, and data not shown). Although consistent with prior studies, GLI1 staining was most prominent in the cytosol [41]. Therefore, GLI1 protein and transcript expression were analyzed by immunohistochemistry and in situ hybridization, respectively, in conjunction with IDH1 R132H staining. As observed for PTCH1, GLI1 expression was detected in a subset of IDH1 R132H-positive cells by sequential antibody staining (Figure 2A and 2B) and by combined in situ hybridization and antibody staining (Figure 2D). The

signal to noise ratio of immunohistochemistry and in situ hybridization was inferior to that of immunofluorescence staining for PTCH1 and IDH1 R132H, however, and the percentages of neoplastic cells expressing GLI1 could not be determined.

SHH transcript expression

The cytosolic expression pattern of SHH in neoplastic cells by immunofluorescence staining (Figure 1C) is consistent with ligand expression in the secretory pathway of a producing cells, as in prior studies SHH staining could not be detected in the cytosol of receiving cells [42]. To test this hypothesis and gain further insight into potential autocrine and paracrine Hh signaling modes, in situ hybridization for SHH transcript was performed in conjunction with GLI1 and PTCH1 expression. Multiplex in situ hybridization revealed SHH and GLI1 transcript expression in separate cells in most, though not all instances (Figure 3A). The close proximity of SHH and GLI1 signal overlying the same cell nucleus in some cases (Figure 3A) suggests a pattern that could represent overlapping cytosolic extensions from separate cells, background signal (Supplementary Figure 4A), or evidence for autocrine signaling. Double immunohistochemical labeling, however, revealed SHH and GLI1 protein expression in separate cells (Figure 3B and Supplementary Figures 3D). In further support that distinct neoplastic cells express Hh ligand and respond to the signal, SHH transcript and PTCH1 protein could be detected in separate, neighboring cells (Figure 3C). As discussed above, these data provide further evidence that an autocrine source of signal is unlikely in cells identified by the presence of Hh transcriptional target expression, GLI1 and PTCH1, and the absence of SHH transcript expression.

Discussion

In these studies IDH1 R132H expression was used to definitively identify neoplastic cells within FFPE patient specimens and characterize the expression patterns of Hh pathway components. By this method we demonstrate that neoplastic cells express SHH ligand, PTCH1 receptor and GLI1 transcription factor. We further demonstrate that PTCH1 and GLI1 are expressed in glioma cells that are predominantly separate, or distinct, from glioma cells that express SHH. As PTCH1 and GLI1 are sensitive Hh gene targets, the absence of SHH staining in the majority of PTCH1- and GLI1-positive glioma cells is incompatible with an autocrine source of SHH ligand. Rather, the expression of SHH in glioma cells that are distinct from those expressing PTCH1 or GLI1 is more consistent with intratumoral paracrine signaling.

Hh signaling contributes to the growth of multiple tumor types, and the therapeutic potential of Hh pathway inhibition may be influenced by whether the pathway is activated in tumor cells or non-neoplastic cells within the microenvironment [14, 43–46]. In a preclinical xenograft model of colon cancer, for example, Hh pathway antagonist treatment delays tumor growth. However, species-specific expression analysis revealed that Hh target genes were down regulated in mouse stroma rather than engrafted human tumor cells [3]. Although the microenvironment serves important roles in supporting tumor growth, the absence of an operational Hh pathway in colon cancer cells may have been one factor contributing to less than favorable clinical trial results obtained with a Hh inhibitor in this

disease [46]. In a primary orthotopic glioma xenograft model, by contrast, Hh pathway antagonist treatment prolongs survival and down-regulates Hh target gene expression in engrafted human cells rather than mouse brain [12]. In conjunction with these findings, the expression of PTCH1 and GLI1 in IDH1 R132H mutant cells demonstrates unequivocally that the Hh pathway is operant in neoplastic glioma cells.

We previously proposed a non-neoplastic source of Hh ligand in gliomas based in part upon observations that Hh ligand expression is not expressed in primary glioma cell cultures, but also on interpretation of SHH staining patterns in glioma specimens without the benefit of a definitive marker for neoplastic cells [10]. At odds with the notion of non-neoplastic cells as the sole source of Hh ligand, however, was the detection of human SHH transcript expression in xenografted WHO grade III gliomas months after xenotransplantation, as non neoplastic neurons from a tumor specimen would not be expected to engraft and survive for that length of time [12]. The detection of SHH ligand in IDH1 R132H cells in patient specimens may reconcile these discrepant findings as, with rare exception, IDH1 mutant cells are not maintained in glioma cultures [47, 48]. Although the findings reported here do not eliminate non-neoplastic cellular sources, the expression of SHH ligand in IDH1 R132H mutant cells clearly indicates that neoplastic cells can serve as a source of ligand in gliomas.

The non-overlapping cellular expression pattern of SHH ligand and Hh pathway transduction components detected by three different methods suggests that autocrine Hh signaling is not the predominant mode of pathway activation in glioma. Rather, the data support the intriguing possibility that Hh signaling occurs between discrete populations of tumor cells. Hh ligands are secreted signaling proteins that during embryonic tissue patterning elicit concentration-dependent cellular responses in adjacent and distant cell types. As in neural tissue patterning, short- and long-range Hh signaling may occur in gliomas. Future studies are warranted to characterize further SHH and PTCH1 and GLI1 expressing cell types to fully elucidate roles of Hh signaling in glioma growth and optimize therapeutic targeting of the Hh pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SHH and PTCH1 expression in IDH1 R132H-mutant glioma cells. Paraffin sections of IDH1 mutant gliomas were analyzed by double immunofluorescence labeling for IDH1 R132H (red) and SHH (green) expression (A–D), IDH1 R132H (red) and PTCH (green) expression (E–H), and SHH (green) and PTCH (red) expression (A–D). Nuclei were counterstained with DAPI (blue). Triple immunofluorescence staining was performed for IDH1 R132H (blue), SHH (green) and PTCH1 (green) expression and analyzed by confocal scanning microscopy (M–P). Arrowheads indicate SHH (white) and PTCH1 (yellow) staining.

Figure 2.

GLI1 expression in IDH1 R132H-mutant glioma cells. (A and B) Paraffin sections of glioma specimens were immunostained for GLI1 (brown; immunohistochemistry) and IDH1 R132H (red, immunofluorescence) and nuclei were counterstained with Hoescht dye (blue). Arrowheads in the insets indicate double-labeled cells in an oligodendroglioma (16772 O). (C and D) Glioma paraffin sections were assayed for GLI1 transcript expression by in situ hybridization. GLI1 expression was detected in a subset of cells (C) that expressed mutant IDH1 protein (D).

Figure 3.

Expression of SHH transcript and pathway components in separate cellular populations. Paraffin glioma specimens were assayed for expression of SHH and the pathway components GLI1 and PTCH1. (A) Multiplex in situ hybridization for SHH (red) and GLI1 (green) transcript expression in an anaplastic oligodendroglioma (12262 AO). Arrowheads indicate GLI1 positive/SHH-negative cells (B) Immunostaining for SHH (brown) and GLI1 (purple) expression in an astrocytoma (13396 A). (C) In situ hybridization for SHH

transcript (red) and immunohistochemistry for PTCH1 protein (brown) expression in an astrocytoma (18033 A). Arrowheads indicate PTCH1 positive/SHH-negative cells.

Table

Tumor	% IDH1 R132H-positive cells expressing SHH (AVG±SEM)	% IDH1 R132H-positive cells expressing PTCH (AVG±SEM)	% SHH-positive cells expressing IDH1 R132H	% PTCH-positive cells expressing IDH1 R132H	% of PTCH-positive cells expressing SHH
12784 O	9.35 ± 1.84	14.9 ± 2.27	95.2	100	2.00
15172 O	7.94 ± 1.54	9.45 ± 3.09	90.0	89.2	13.3
16273 A	0.501 ± 0.335	19.4 ± 2.77	18.2	94.0	0.000
14657 A	16.1 ± 2.15	9.22 ±2.53	100	30.5	3.85