Combined Expression of Nestin and SPARC Identifies In Situ Tumor Cells in Astrocytic Tumors of all Grades

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Abstract

Background: Malignant gliomas are heterogeneous, diffuse and invasive by nature. Histopathological identification of glioma tumor cells is mandatory to characterize the tumors and the extent of infiltration in the surrounding normal parenchyma.

Methods: In order to identify specific markers for tumor cells not expressed in non-neoplastic brain tissues, we used a well-annotated tissue microarray (TMA) containing 45 samples from patients that suffered from several subtypes of low grade and high grade gliomas (pilocytic astrocytoma, oligoastrocytoma, low grade astrocytoma, anaplastic astrocytoma, primary and secondary glioblastomas), non-gliial tumors (medulloblastoma and metastasis) as well as fetal, epileptic, and gliotic specimens used as non-tumoral control tissues. The TMA was assessed for 24 proteins involved in tumor proliferation, migration, invasion, and differentiation, or acting as transcription factors and metabolic enzymes.

Results: This immunohistological analysis revealed that nestin and secreted protein, acidic and rich in cysteine (SPARC) are expressed in tumor cells in all glioma subtypes and developmental tissues but rarely in mature epithilic tissue. In addition to these two markers, the expression of mutated isocitrate dehydrogenase 1 (IDH1(R132H)) also identified tumor cells but only in some subtypes of gliomas.

Conclusions: Taken together, our data suggest that the combination of nestin and SPARC expression characterizes tumor glioma cells. These proteins may represent relevant glioma immunohistological markers that might be molecular targets in glioma therapy.

Keywords: Glioma; Tissue microarray; Nestin; IDH1; SPARC

Introduction

Gliomas are a heterogeneous group of neuroectodermal tumors that arise from the glia. Despite the huge technical development of diagnostic and surgical procedures, the prognosis for patients with gliomas, and particularly glioblastoma multiforme (GBM), remains poor. The survival of these patients does not exceed more than few months (11 to 16 months) after surgical resection and supplemental treatments including chemotherapy and radiation therapy [1,2]. Therefore, malignant gliomas are considered as a great challenge for neurosurgery because of their invasive and propagation behaviors, high recurrence rates and substantial morbidity. Low-grade gliomas are usually encountered in younger patient populations between the third and fourth decades of life [3] and most patients survive a decade or more.

Only 30% of radiologically suspected low-grade gliomas are histopathologically classified as malignant gliomas. Such discrepancies between radiological and histopathological diagnosis indicate that imaging alone is not sufficient in predicting the status of brain tumors. Therefore, a histopathological approach is mandatory in any case of suspected glioma to provide diagnosis, prognosis and prediction of treatment for brain tumors.

Many studies have described over 60 genetic alterations such as mutations, amplifications, and deletions that cause proliferation, infiltration as well as resistance to standard treatments of these tumors [4-6]. The most common changes are amplification of the MDM2, PDGFR, CDK4 genes, mutations of epidermal growth factor receptor (EGFR), isocitrate dehydrogenase 1 (IDH1), PTK, PIK3CA, and deletion of TP53, RB1, and CDKN2A. Combination of these alterations contributes to the three major pathways PI3K, p53 and RB [7]. There has been considerable interest in the recent years in classifying GBM on the basis of gene expression [1,8]. Genetic features can be associated with each type of tumor, but there are still overlaps between groups. For example, in primary GBM, that arises de novo, epithelial growth factor receptor (EGFR) amplification but no IDH1 mutation are described, whereas in secondary GBM, in which pre-existing low-grade glioma was observed, PDGFR amplification, and mutations in IDH1 (on arginine R132), TP53 and RB1 are frequent [9].

Recently, a subpopulation of glioma cells with stem cell characteristics has been described and identified as the tumor initiating cells and the main cause for tumor infiltration, recurrence and therapy-resistance [10,11]. These tumor cells can be identified by markers specific for stem cells such as the membranous glycoprotein CD133, the transcription factor SOX2 or the cytoskeletal protein nestin. Nestin is down-regulated after development but is re-expressed in the adult organism under certain pathological conditions such as brain injury, ischemia, inflammation and neoplastic transformation [12].

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Received January 28, 2015; Accepted February 20, 2015; Published February 22, 2015.


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In order to identify specific biomarkers for tumor cells in gliomas, we performed a comparative immunohistological study using a Tissue MicroArray (TMA) containing human tissues isolated from non-neoplastic brains (epileptic, gliotic and fetal brain samples), low grade gliomas (pilocytic astrocytoma (PiA), low grade astrocytoma (LGA), and oligoastrocytoma (OA)), anaplastic astrocytoma (AA), primary GBM (pGBM), secondary GBM (sGBM), and other tumor tissues (medulloblastoma (Med), and brain metastases (Meta)).

Material and Methods

Human tissue samples constituting the Tissue MicroArray (TMA)

All human brain tissue samples were obtained according to the guidelines of the Ethical Committee of Bern, Switzerland, and with informed consent obtained from each patient. The TMA was constructed with paraffin-embedded punched tissue samples (1 mm of diameter) from 45 histologically representative cases of different subtypes of gliomas of various WHO grades [13] (LGA: n=7; OA: n=3; PiA: n=3; AA: n=9; pGBM: n=17; sGBM: n=6), 10 non-neoplastic cases (epileptic tissue: n=4; fetal tissue: n=3; gliotic tissue: n=3) used as control reference tissues and additional 6 other CNS tumor cases (Med: n=5; Meta: n=1). Some tumors were represented by 5 samples forming an invasion gradient (3 LGA, 3 OA, 3 PiA, 3 AA, 8 pGBM, 3 sGBM, 2 Med), each separated by 2-4 mm from the center to the periphery of the tumor, in order to visualize potentially infiltrative tumor cells into the surrounding normal appearing brain parenchyma. This TMA was used as a template for immunohistochemistry to identify specific biomarkers for gliomas.

Immunohistochemistry

The antibodies used for immunohistochemistry are given in Table 1. Prior to incubation with the primary antibody, 2-3 micrometers paraffin-embedded sections were dewaxed, rehydrated, and pretreated as indicated in Table 1. Sections were then (and following all subsequent steps) washed in Tris-buffered saline (TBS) and incubated with the primary antibody diluted in TBS with 0.5% casein and 5% normal goat serum, for 60 min at room temperature. Next, depending on the species in which the primary antibody had been raised, either a 1:300 dilution of a biotinylated goat-anti-rabbit Ig antiserum (Dako, Glostrup, Denmark) was applied for 45 min. Thereafter sections were incubated either with an avidin-biotin-complex/horseradish peroxidase (1:100 in TBS) (Vector, Burlingame CA, USA) for 45 min and developed with a 30% hydrogen peroxide (H2O2, Sigma, St. Louis MO, USA) or with 0.005% H2O2, or with a streptavidin-biotin-complex/alkaline phosphatase (1:200 in TBS) (Dako) for 45 min and developed with new fuchsin–naphtol AS-BI (Sigma, St. Louis MO, USA) for 30 min. For nestin and SPARC quantification, positive cells and processes were counted, with the exclusion of the microvascular-associated cells. The number of positive cases/cells was expressed as percentage of total number of cases/cells. In Table 2, H+ represents a number of positive cells >50%, L+ represents a number of positive cells < or equal to 50%.

Results

Pre-selection of potential glioma markers from the TMA

The TMA was assessed for proteins involved in tumor proliferation, migration, invasion, and differentiation, or acting as transcription factors and metabolic enzymes (Figure 1). Throughout the 24 immunohistological stainings some proteins were detected in most cells in all tissue samples including normal non-neoplastic tissues such as PCNA, TN-C, substance P, and MAP2 (Figure 2). These proteins could not be considered specific for tumor cells due to their strong expression in controls tissue samples. In contrast, other proteins were expressed in the tumor samples only, with no or rare immunopositive cells present in non-tumoral control tissues. The quantification of positive cells confirmed that nestin, SPARC and IDH1(R132H) were potential markers for tumor cells. These three markers were then selected for further analysis and quantification in each tumor type present on the TMA (Table 2).

Expression of Nestin, SPARC and IDH1(R132H) in normal brain tissues

Immunohistochemical staining for nestin revealed numerous nestin-positive cells in fetal tissues (F, about 50% of the cells, Figure 3), but not in epileptic tissues (E), except one single cell (Figure 3, inset), and the presence of nestin-positive cells associated with the microvasculature (Figure 3, white arrows). Nestin was detected on some reactive astroglial cells in the gliotic tissue (G, 15% of the cells, Figure 3, black arrows), in addition to positive microvascular cells (Figure 3, white arrow). These observations emphasize the expression of nestin under certain circumstances such as proliferation, tissue remodeling (plasticity), or astrocytic activation.

In fetal tissues, SPARC was present in microvessels and was also observed in 25% of the cells as well as in the extracellular parenchyma (Figure 3, white arrow). Some SPARC-positive cells were observed in epileptic tissues (25% of cells, Figure 3, black arrows), in addition to some positive cells associated with the microvasculature (Figure 3, white arrow). In gliotic tissue, some rare glial cells expressed SPARC (15% of the cells, the same cells that are expressing nestin, Figure 3, black arrow), as well as some cells associated with microvessels (Figure 3, white arrow).

No expression of IDH1(R132H) was observed in these three non-tumoral tissue samples (Figure 3).

Expression of Nestin, SPARC and IDH1(R132H) in brain tumors

Low grade gliomas: Immunohistochemistry revealed the presence of nestin-positive cells in all 13 cases of PiA and OA (in 90% and 50% of cells, respectively, Figure 4), and 90% LGA (Figure 5). In OA, mostly astrocytes expressed nestin, with neoplastic oligodendrocytes showing no staining. Nestin-positive cells were also found in the periphery, as illustrated for the case of OA (Figure 4p) and for the 2 cases of LGA (Figure 5p). No sample from the periphery was available for PiA.

Semiquantification of immunopositive signals

Digital images from the tumor centers were captured with a Leica DMRE microscope set with 40x objective (total magnification 400x) coupled to a ColorView III high resolution CDD color camera, and multiple images (6) were merged in order to reconstitute the whole sample punch section. ImageJ image processing program with manual quantification was used to quantify the total number of cells detected by their nuclei and numbers of cells positive for the specific markers. For nestin and SPARC quantification, positive cells and processes were counted, with the exclusion of the microvesSEL-associated cells. The number of positive cases/cells was expressed as percentage of total number of cases/cells. In Table 2, H+ represents a number of positive cells >50%, L+ represents a number of positive cells < or equal to 50%.
Similar observations were done for SPARC. SPARC-positive cells were present in all cases of low grade glioma (PiA, OA and LGA), with about 90% of positive cells in the tumor center but also in the periphery of the tumor (Figure 4 and 5c and 5p).

IDH1(R132H) immunopositivity was found in none of the PiA cases, in 75% of cells in all 3 cases of OA (Figure 4 and Table 2), and in 90% of cells in 5 out of 7 cases of LGA (Figure 5 and Table 2). In OA and the positive cases of LGA, IDH1(R132H)-positive cells were also present in the periphery (Figure 4p and 5 case 1, 5p).

Anaplastic astrocytoma: IHC analysis of AA showed nestin, as well as SPARC expressed in 80- to 90% of cells in most of the cases analysed, whereas 7 out of 9 cases were IDH1(R132H) immunopositive (Figure 6 and Table 2). Cells positive for all the three markers were found also in the periphery of the tumors (Figure 6, case 1, 6p). Interestingly, in IDH1(R132H)-negative cases, Nestin and SPARC were expressed in 80- to 90% of cells in most of the cases as well as SPARC expressed in 80- to 90% of cells in most of the cases analysed, whereas 7 out of 9 cases were IDH1(R132H) immunopositive (Figure 6 and Table 2). Cells positive for all the three markers were found also in the periphery of the tumors (Figure 6, case 1, 6p).

Table 2: Number of positive cases for nestin, SPARC and IDH1(R132H) in the different control brain samples and the brain tumor samples.

<table>
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<th>L+</th>
<th>Neg</th>
<th>%+</th>
<th>SPARC H+</th>
<th>L+</th>
<th>Neg</th>
<th>%+</th>
<th>IDH1(R132H) H+</th>
<th>L+</th>
<th>Neg</th>
<th>%+</th>
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Abbreviations: F: fetal tissue; E: epilepsy tissue; G: gliotic tissue; PIA: pilocytic astrocytoma; OA: oligoastrocytoma; LGA: Low-grade astrocytoma; AA: anaplastic astrocytoma; pGBM: primary glioblastoma; sGBM: secondary glioblastoma; Med: medulloblastoma; Meta: metastasis. n: number of cases, H+: >50% of positive cells; L+: < or equal to 50% positive cells; Neg: negative, no positive cells; %+: percentage of positive cases.

Table 1: Primary antibodies used for immunohistochemistry and technical information.

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<th>Concentration</th>
<th>Pretreatment</th>
<th>Technique</th>
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<td>HRP-DAB</td>
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<tr>
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<td>abcram</td>
<td>1 :1600</td>
<td>Microwave, citrate</td>
<td>HRP-DAB</td>
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<td>R&amp;D Systems</td>
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<td>AP-NF</td>
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Manufacturer locations: abcam, Cambridge, UK; BD Biosciences, Basel, Switzerland; BioGenex, San Ramon, CA, USA; Cell Signaling, Beverly, MA, USA; Dako, Glostrup, Denmark; Dianova, Hamburg, Germany; Leica, Nüningen, Switzerland; Millipore, Merck Millipore, Darmstadt, Germany; Novocastra, Newcastle-upon-Tyne, UK; R&D Systems, Minneapolis, MN, USA; Serotec, Düsseldorf, Germany; Sigma-Aldrich, St-Louis, MO, USA; Ventana Medical Systems, Tucson, AZ, USA.

Techniques: AP-NF: ABC method using alkaline phosphatase and new fuchsin; AEC: 3-amino-9-ethylcarbazole; HRP-DAB: ABC method using horseradish peroxidase and 3,3-diaminobenzidine (DAB) or OptiView DAB detection kit/Benchmark XT (Ventana Medical Systems).
Primary GBM: Nestin-positive cells, as well as SPARC-positive cells (90% of cells for both markers) have been detected in all 17 cases of pGBM (Table 2). SPARC showed intra- and extracellular expression profiles in the center of the tumor (Figure 7, upper part, c). Of note, none of these pGBM showed IDH1(R132H) expression. The expressions of Nestin and SPARC were very strong and delineated clear margins between the tumor mass and the infiltration zone, however, a small number of cells expressing Nestin and SPARC were also observed in the periphery of the tumors (Figure 7, upper part, p).

Secondary GBM: Out of the 6 sGBM cases, we observed strong staining for nestin in 3 cases, in 2 cases for SPARC (80 to 90% of the cells), as well as in 4 cases for IDH1(R132H) (90% of cells) (Figure 7, lower part, c). In these cases, nestin, SPARC and IDH1(R132H) were also expressed in cells in the infiltration zone of the tumors (Figure 7, lower part, p).

Two cases did not express SPARC in the tumor, or only in very rare astrocytes. Out of these 2 cases, one showed a low expression of Nestin (case 1, Figure 8), and the other strongly expressed nestin (case 2 in Figure 8). IDH1(R132H) was strongly expressed in both cases (Figure 8). In these 2 cases, a high cellularity was observed, and cells were expressing the proliferative cell nuclear antigen (PCNA), and
p53, reflecting the presence of loss-of-function mutation of this anti-tumoral protein (Figure 8). Images from case 2 probably represent the so-called “hot-spots” of proliferative cells. Considering the great morphologic heterogeneity of GBM, it is possible that the samples taken from case 2 hit such hot spots of IDH1(R132H)-positive tumor cells that clearly express nestin, as well as proliferation markers, but not SPARC, indicated by the black arrows in Figure 8. This suggests also that highly proliferative tumor cells do not express SPARC, and that this marker may be expressed during an earlier or later phase of tumor progression.

Expression of Nestin, SPARC and IDH1(R132H) in other tumor types

**Medulloblastoma:** Almost no IDH1(R132H) staining was observed in medulloblastoma samples, but they all show SPARC expression, 3 cases in more than 75% of the cells, and 2 showing lower expression (Figure 9, upper part). Moreover, extracellular SPARC was also found in the parenchyma in all high positive cases. In contrast to gliomas, nestin-positive cells were mainly observed in the tumor vasculature, and only some isolated cells expressed nestin in the parenchyma (25% of cells) in all cases.

**Metastasis:** In the single case of brain metastasis, immunolabeling of pseudo-rosettes surrounding blood vessels were observed (Figure 9, lower part). Nestin was strongly expressed by endothelial cells, but tumor cells constituting the rosettes showed less intense nestin immunoreactivity, and some nestin-positive tumor cells outside the cell clusters were also observed (25% of cells). In this metastasis, 50% of cells strongly expressed SPARC. This protein was also expressed in vascular cells located at the center of the rosettes. All the cells in the tumor were IDH1(R132H) negative.

**Discussion**

Among the 24 proteins stained in our TMA, IDH1(R132H), nestin and SPARC were clustered in most primary and secondary brain tumors. They were then further analysed in order to identify their potential as specific markers for tumor cells. These markers were concentrated in the center of the tumors, and expressed also by tumor cells infiltrating normal tissue.

Nestin belongs to class VI intermediate filaments and is expressed in proliferating cells during the developmental stages in a variety of embryonic and fetal tissues. During development, nestin is produced

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**Figure 2:** Quantitative analysis of each individual marker. **A:** Quantification of positive cells for each marker in control and tumor tissue samples, expressed as % of the total number of cells in the region of interest. Three markers were highly expressed in most of gliomas and absent in normal controls and less frequent in other tumors: nestin, SPARC and IDH1(R132H). **B:** General views of the TMA showing tissue samples represented by 5 sections each (when available). The comparison of the staining of the three selected markers shows co-expression of nestin and SPARC in numerous samples, whereas a smaller number of samples express IDH1(R132H). The non-tumoral control cases (F, E and G) are located in the frames.
Nestin expression is used as a marker of proliferating and migrating cells, as well as in brain tumors including GBMs [21,22]. Indeed, all tumor cases analysed in the present study, including medulloblastoma and metastasis, showed various levels of cells expressing nestin. The higher expression level of nestin in gliomas of astrocytic lineage (90% of tumor cells in PiA, LGA, AA) compared to OA (only 50% of tumor cells) illustrates the specificity of nestin for astrocytoma cells and not for oligodendrocytes. In astrocytic gliomas, an association between

in neuronal and glial stem/progenitor cells in the mammalian CNS and is consecutively replaced by other intermediate filament proteins (neurofilaments or GFAP). Nestin has also been shown to be expressed in other cell types such as endothelial cells of capillaries [14], smooth muscle cells and pericytes in the CNS capillaries [15,16], but not in larger blood vessels. This is in accordance with our observations that most of the capillaries were nestin-positive, even in the normal, non-tumoral tissues. Interestingly, a rapid re-expression of nestin was found in reactive astrocytes of the adult rodent brain after seizures [17], trauma [18] and brain ischemia [19,20], reflecting the reversal of astrocytes to a more immature phenotype during the reactive state. We observed only one single cell expressing nestin in our epilepsy samples that may constitute a glia cell involved in plasticity.
Secreted SPARC regulates extracellular matrix, and matrix metalloproteinases activity, modulates cell growth, cell morphology and migration responses [25-27]. Intracellular SPARC, however, might have different functions in glioma, such as differentiation and survival [28]. Through the CNS, SPARC is highly localized in mature ramified microglia. Following ischemic and excitotoxic injury, reactive perilesional astrocytes upregulate the expression of SPARC [29]. Accordingly, we found reactive astrocytes expressing intracellular SPARC in gliotic samples.

Interestingly, SPARC has been shown to induce cell cycle arrest in tumor cells [30,31] and to functionally contribute to glioma invasion in vitro and in vivo [32-34]. Indeed, it is well known that migration and proliferation are antagonistic cellular behaviors. In a model of glioma xenographs, the patterns of invasion and the extent of growth delay correlated with the level of SPARC expressed. It has been proposed therefore that the ability of SPARC to promote invasion depends on the level of its secretion and the resultant modulation of the level of adherence and motility induced [32]. We observed no SPARC in highly proliferating stem-like cells located at proliferation "hot spots" in 2 cases of sGBM, but the presence of extracellular SPARC in numerous cases of fetal tissue and in gliomas, in which remodeling and invasion are active. Therefore, this observation is in accordance with the “go or growth” theory illustrating the fact that factors promoting glioma invasion delay cell cycle and tumor growth. Therefore, targeting SPARC may represent an anti-invasion therapeutic way for malignant gliomas, together with an anti-proliferative agent.

IDH1 mutations are the earliest detectable genetic alterations in low-grade diffuse astrocytomas and oligodendrogliomas, suggesting that the developmental pathways of these tumors are distinct from those of pGBM. Secondary GBMs progress from precursor low-grade diffuse astrocytoma or anaplastic astrocytoma. Histologically, primary and secondary GBMs are largely indistinguishable, but they differ in their genetic and epigenetic profiles. Our results confirm that IDH1(R132H) mutation recognizes most LGG and resulting sGBMs, whereas in strictly absent in pGBMs [35].

Taken together, our results indicate that nestin and SPARC are co-expressed in most brain tumor cells, highlighting the full extent of the tumors: the core and the infiltration zone. Nestin is expressed in all brain tumors, particularly in cell populations with robust proliferative, clonogenic and tumorigenic capacity, supporting its potential value as marker for neoplastic cells. This finding might be helpful to detect tumor cell infiltrations in the peripheral margin of gliomas, eventually also in cell processes. In contrast, SPARC is expressed also in non-tumoral tissue, but is low in some sGBM, in which active tumor cell proliferation at the "hot spots" is observed. Moreover, its presence in normal cerebral samples such as epileptic tissue makes SPARC a less specific marker than nestin.

IDH1(R132H) is the gold-standard marker for the diagnosis of mutation-bearing gliomas, and represents the most sensitive marker differentiating pGBM from sGBM. We suggest that these three proteins, nestin, SPARC and IDH1(R132H), taken together, are potential markers for brain tumor cells. The need for tumor specific-targeted therapies is one major therapeutic challenge for the future. The understanding of the molecular pathways and genetic complexity of various subtypes of gliomas has increased the number of novel glioma therapeutic strategies, such as gene therapy, stem cell-based or nanoparticle-based therapies [36]. However, the identification of common markers specific for all gliomas and other brain tumors such as medulloblastoma may simplify the development of a single
therapeutic approach in glioma treatment. In this regard, nestin and SPARC may constitute potential brain tumor biomarkers.

Acknowledgments

We thank Prof. Stephan Frank, Institute of Neuropathology Basel for neuropathological descriptions of the cases, David Friedrich and Monika Bänziger, Institute of Neuropathology Basel, for help with the IHC protocols.

References


