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High expression of the stem cell marker nestin is an adverse prognostic factor in WHO grade II-III astrocytomas and oligoastrocytomas

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Abstract

Infiltrating astrocytomas and oligoastrocytomas of low to anaplastic grade (WHO grades II and III), in spite of being associated with a wide range of clinical outcomes, can be difficult to subclassify and grade by the current histopathologic criteria. Unlike oligodendrogliomas and anaplastic oligodendrogliomas that can be identified by the 1p/19q codeletion and the more malignant glioblastomas (WHO grade IV astrocytomas) that can be diagnosed solely based on objective features on routine hematoxylin and eosin sections, no such objective criteria exist for the subclassification of grade II-III astrocytomas and oligoastrocytomas (A+OA II-III). In this study, we evaluated the prognostic and predictive value of the stem cell marker nestin in adult A+OA II-III (n=50) using immunohistochemistry and computer-assisted analysis on tissue microarrays. In addition, the correlation between nestin mRNA level and total survival was analyzed in the NCI Rembrandt database. The results showed that high nestin expression is a strong adverse prognostic factor for total survival (p=0.0004). The strength of the correlation was comparable to but independent of the isocitrate dehydrogenase 1/2 (IDH 1/2) mutation status. Histopathological grading and subclassification did not correlate significantly with outcome, although the interpretation of this finding is limited by the fact that grade III tumors were treated

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more aggressively than grade II tumors. These results suggest that nestin level and IDH 1/2 mutation status are strong prognostic features in A+OA II-III and possibly more helpful for treatment planning than routine histopathological variables such as oligodendroglial component (astrocytoma vs. oligoastrocytoma) and WHO grade (grade II vs. III).

Keywords

mixed glioma; outcome; prognosis; progression-free survival; low-grade gliomas; anaplastic gliomas

Introduction

Infiltrating astrocytomas (A) and oligoastrocytomas (OA) of low to anaplastic grade (WHO grades II and III) constitute a diagnostically challenging group of tumors with a highly variable clinical course. The main reason why the histological subclassification and grading (A vs. OA, grade II vs. III) of these tumors is difficult is the vagueness and subjectivity of the criteria, which has led to poor interobserver agreement [1, 2]. While the 1p/19q codeletion is helpful in the diagnosis of pure oligodendrogliomas (O II-III), this genetic alteration is absent in grade II-III astrocytomas and oligoastrocytomas (A+OA II-III). Hence the search for novel, objective prognostic and predictive markers for A+OA II-III is of paramount clinical importance.

In this study, we investigated the prognostic and predictive value of nestin, a type VI intermediate filament protein that is expressed by primitive neuroepithelial cells and neural precursor cells during development [3-12]. In most mature central nervous system cells, nestin expression is downregulated, but re-expression of nestin can be observed gliomas [5, 9, 13-16]. Prior studies have reported higher levels of nestin expression in glioblastomas (GBMs; WHO grade IV astrocytomas) compared with lower-grade gliomas (WHO grades II-III) [4-6, 12]. It has also been reported that higher nestin expression is associated with shorter survival when GBMs (grade IV) and anaplastic (grade III) or low-grade infiltrating gliomas (grade II) are combined into a single group [6, 12, 13, 16-18]. However, the prior studies did not correct for the effect of tumor grade by multivariate analysis. Such studies cannot determine the clinical usefulness of nestin as a prognostic marker, because it is already known that GBMs are much more malignant than lower grade gliomas. Any effect on survival seen in a combined group of grade II-IV gliomas could be due to nestin expression being higher in GBMs. This possibility was not ruled out in the previous studies. The histopathological diagnosis of GBM is made based on the presence of necrosis or microvascular proliferation on routine hematoxylin & eosin (H&E) sections [19]; therefore, the use of additional prognostic markers correlating only with the diagnosis of GBM would be of limited benefit.

Recently, the presence of acquired isocitrate dehydrogenase 1/2 (IDH 1/2) mutations in tumor cells has emerged as a strong favorable prognostic factor in grade II-III gliomas [20-23]. In this study, we analyzed the IDH 1/2 mutation status by immunohistochemistry and DNA sequence analysis, nestin expression level by immunohistochemistry and computer-assisted image analysis on tissue microarrays (TMAs), and clinical outcome in

grade II-IV gliomas, with a focus on A+OA II-III. The effect of nestin expression on clinical outcome was evaluated using multivariate analysis, with the goal of finding an objective way to stratify the A+OA II-III group of tumors by clinical outcome.

Methods

Cases

The study included 50 A+OA II-III cases (22 grade II and 28 grade III) diagnosed at UT Southwestern Medical Center in 1997-2010. Of these, 42 (84%) had an IDH1 or IDH2 mutation, as determined by immunohistochemistry for IDH1 R132H followed by sequence analysis of the IDH1 and IDH2 genes using a highly sensitive method (see below) in the tumors that were negative by immunohistochemistry. OAs (n=31), all negative for 1p/19q LOH, were preferentially selected for this study. No tumor had EGFR amplification as determined by chromogenic in situ hybridization (CISH) for the EGFR gene. All histological diagnoses were re-reviewed according to current WHO criteria by the same, experienced tumor neuropathologist (KJH) for consistency. The age range at diagnosis was 20-66 (median, 37.5). Twenty-four of the patients were women and 26 were men. A gross total resection was achieved in 35 cases, whereas the remaining patients underwent a subtotal resection (n=10), open biopsy (n=4), or stereotactic needle biopsy (n=1). Adjuvant therapy was concurrent temozolomide and radiation (TMZ+rad; n=20), chemotherapy or radiation alone (n=20), none (n=9), or unknown (n=1). Median follow-up was 4.3 years overall and 5.1 years among the 36 surviving patients. For comparison, glioblastomas (GBMs; n=77) and 1p/19q-codeleted grade II-III oligodendrogliomas (O II-III; n=22) were included in some analyses. The study was approved by the UT Southwestern Medical Center Institutional Review Board (UTSW IRB CR00003359/STU 022011-081). For independent confirmation, we also analyzed data on A+OA grade III in the NCI Rembrandt database, which contains anonymized data on gliomas diagnosed from 1991 to 2004 [24] (<https://caintegrator.nci.nih.gov/caintegrator/>). Due to the restriction on the types of analyses that can be performed using the Rembrandt web interface, the analysis was performed on Rembrandt raw data. The Rembrandt raw data on nestin expression and clinical variables can be obtained by requesting it by email (ncicb@pop.nci.nih.gov).

IDH1 and IDH2 sequence analysis

The IDH1 and IDH2 sequence analysis method was based on a previously published fluorescence melting curve analysis method [25] but optimized for greater sensitivity, which allowed the analysis of small specimens such as stereotactic brain biopsies. Tissue sections were deparaffinized by two xylene and ethanol washes. Areas containing tumor tissue were dissected from the sections. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germantown, MD). Melting curve analysis was performed on a LightCycler 1.2 (Roche, Indianapolis, IN) using previously published hybridization probes [25] and lab-developed primers (Supplemental Table 1). The 20 µL IDH1/IDH2 multiplex reaction contained 2.0 µL 10x Titanium Taq buffer, 250 µmol/L dNTPs, final MgCl₂ concentration of 3.0 mmol/L, 0.25 µL Titanium Taq polymerase (Clontech, Mountainview, CA), 0.50 µmol/L each of primers ID1PF2 and ID1PR4 (for IDH1) and ID2PF2 and ID2PR3 (for IDH2), 0.2 µmol/L each of four fluorescent IDH1/IDH2 hybridization probes,

and 10-40ng sample DNA. Real-time PCR cycling conditions were 95 °C for 1 min denaturation followed by 20 cycles of pre-amplification (95 °C for 10 sec, 55 °C for 10 sec, 72 °C for 15 sec) and 40 cycles of fluorescence detection (95 °C for 5 sec, 54 °C for 6 sec, 72 °C for 15 sec). Melting curve analysis was run from 45 to 95 °C at a rate of 0.1 °C /sec with continuous fluorescence reading.

Conventional Sanger sequencing was performed as follows to confirm all detected mutations. The 25 µL PCR reactions each contained 2.5 µL 10x AmpliTaq Gold 10x buffer, 200 µmol/L dNTPs, final MgCl₂ concentration of 3.0 mmol/L, 1.25 units AmpliTaq Gold polymerase (Life Technologies, Carlsbad, CA), 0.40 µmol/L each of primers ID1PF4 and ID1PR4 (for IDH1) or ID2PF1 and ID2PR1 (for IDH2), and 5-20ng sample DNA (Supplemental Table 1). Thermocycler settings were the same for the IDH1 and IDH2 reactions: 10 min denaturation at 95 °C followed by 45 cycles of 95 °C for 10 sec, 54 °C for 15 sec, 72 °C for 40 sec, with a final 72 °C step for 10 min. Amplicons were cleaned with a Qiagen QIAquick PCR Purification column and 10-30 ng of the eluted DNA was added to a 10 µL Sanger sequencing reaction that contained 2.0 µL of 5x BigDye buffer, 3.0 µL of BigDye Terminator v3.1 Cycle Sequencing Mix (Life Technologies, Carlsbad, CA), and 0.32 µmol/L of sequencing primer ID1SF1 (for IDH1) or ID2PR3 (for IDH2) (Supplemental Table 1). Thermocycler conditions were 96 °C for 2 min, followed by 35 sequencing cycles (96 °C for 10 sec, 54 °C for 5 sec, and 60 °C for 4 min). Products were electrophoresed on a 3130xL Genetic Analyzer and manually read.

Tissue microarrays and immunohistochemistry

Nestin protein expression was evaluated on tissue microarrays by immunohistochemistry. Cores (1.0 mm) for tissue microarrays (TMAs) were punched using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). The donor blocks were formalin-fixed, paraffin-embedded tissue blocks of human gliomas. TMA sections of human gliomas were cut at 4 micron thickness and deparaffinized. For antigen retrieval, the sections were heated for 30 min at 98 °C in CC1 Solution (Ventana Medical Systems, Tucson, AZ). The sections were exposed to a rabbit polyclonal antibody to human nestin (AB5986; Abcam) at a dilution of 1:1,000. The signal was developed with ultraView universal DAB detection system (Ventana). The immunostaining procedure was performed using the Benchmark XT automated stainer (Ventana).

Computer-assisted image analysis

The immunostained TMA slides were scanned with a Nanoscope microscopic slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). A semiquantitative estimate of nestin protein level per tumor cell was obtained by measuring the ratio of the area covered by the brown chromogen corresponding to nestin and the area covered by the hematoxylin counterstain corresponding to tumor cell nuclei (Fig. 1). The image analysis was performed using Imagescope software, version 11.1.2.752 (Aperio, Vista, CA).

Statistics

Nestin levels were dichotomized to high and low at the median. Multivariate regression was performed using a Cox proportional hazards model where all variables were entered in the

model and then the variables with $p > 0.1$ were removed sequentially. The software was Medcalc version 12.3 (Medcalc, Ostend, Belgium). Kaplan-Meier survival curves were generated and compared with the Mantel-Cox (logrank) test using GraphPad Prism version 6.01 software (GraphPad, La Jolla, CA). The following covariates were included in multivariate survival analyses (Cox proportional hazards model): nestin, IDH 1/2 mutation status, WHO grade, oligodendroglioma component (astrocytoma or oligoastrocytoma), MIB-1 proliferation index, age at diagnosis, sex, extent of resection (gross total vs. partial or biopsy), and type of adjuvant therapy (combined temozolomide and radiation vs. other). Univariate analyses were performed by comparing Kaplan-Meier curves (logrank test).

Results

Nestin protein level by histological classification and WHO grade

The average nestin level was similar in astrocytomas (A) and oligoastrocytomas (OA) as well as in WHO grade II and WHO grade III tumors (supplemental Fig. S1). Nestin protein level was higher in GBM compared with A+OA II-III and O II-III ($p < 0.0001$) (Fig 2). As expected, the GBM group was also associated with the shortest total survival and progression-free survival among the three groups of gliomas (supplemental Figs. S2 and S3). Further analyses were limited to the A+OA II-III group.

Nestin protein level and clinical outcome in A+OA II-III

In the A+OA II-III group of tumors, nestin protein level ($p = 0.0022$) and IDH1/2 mutation status ($p = 0.0035$) were the strongest prognostic factors for total survival in multivariate analysis (Table 1). Both nestin ($p = 0.0004$) (Fig. 3) and IDH1/2 mutation status ($p = 0.0006$) were also significantly associated with total survival in univariate analyses. In addition, nestin protein level and IDH 1/2 mutation status were weak predictive factors for progression-free survival ($p < 0.05$). When the tumors were stratified by both nestin level and IDH1/2 mutation status, the longest total survival was seen among patients with low nestin and IDH1/2 mutations whereas the shortest survival was among patients with high nestin and no IDH1/2 mutations; the remaining two combinations were associated with intermediate survival ($p < 0.0001$; supplemental Fig. S4).

The publicly available survival and gene expression data in the NCI Rembrandt database contained 47 patients diagnosed with grade III astrocytomas and oligoastrocytomas in 1985-2004. Analysis of nestin mRNA level in these tumors indicated an inverse relationship with total survival ($p = 0.004$) (Fig. 4), thus providing independent confirmation of our results.

Practical guidelines for evaluating nestin level when image analysis is not available

In general, tumors with higher than median expression of nestin by computer scoring corresponded to tumors where 10% or more of the tumor cells were estimated to be positive for nestin by manual scoring. In the tumors where 10% or more of the cells were positive, the staining intensity was almost always estimated to be moderate or strong by manual scoring, whereas weak staining intensity was typically seen in the tumors where fewer than 10% of the cells were positive. Thus, from the practical standpoint, if computer-assisted

image analysis is not available, positivity of 10% or more of the tumor cells at moderate or strong staining intensity by manual scoring corresponds to high nestin expression. As an example, glioma #3 in Fig. 1 is near the lower end of the spectrum for what was considered high nestin expression in this study.

Histopathological classification and clinical outcome in A+OA II-III

Within the A+OA II-III group of tumors, histopathological grading (grade II vs. III) and subclassification (astrocytoma vs. oligoastrocytoma) had no significant effect on total or progression-free survival in multi-or univariate analyses ($p>0.1$).

Discussion

We found that high nestin protein expression is a strong adverse prognostic factor in A+OA II-III, a group of gliomas that is otherwise difficult to subclassify using routine histology (Fig. 3). Although previous studies have shown nestin expression to be a significant prognostic factor in mixed groups of tumors containing GBMs [6, 12, 13, 16-18], our study is the first to focus on low and intermediate grade infiltrating gliomas and to show the clinical relevance and independence of nestin level as a prognostic factor by multivariate survival analysis. Multivariate survival analyses also showed that the effect of nestin level on total survival is independent of IDH 1/2 mutation status and equally strong (Table 1). Evaluation of publicly available data in the NCI Rembrandt brain tumor database on gene expression and clinical outcome provided independent confirmation of our results (Fig. 4). The effect of nestin level and IDH 1/2 mutation status on total survival was stronger than that of routine histopathological subclassification and grading according to the WHO criteria. Thus, our results suggest that nestin level and IDH 1/2 mutation status may be more helpful in clinical risk stratification and treatment planning than reliance on routine histopathological variables such as oligodendroglial component (astrocytoma vs. oligoastrocytoma) and WHO grade (grade II vs. III).

Evidence from cell culture studies and animal models support a role for nestin as a tumor promoter. Inhibition of nestin expression by nestin siRNA reduced cell growth in cultured astrocytoma cells [9]. Neural stem cells in a zebrafish nestin knockdown model showed a markedly impaired rate of self-renewal that was related to an increased rate of apoptosis [26]. Nestin is normally expressed by primitive neuroectodermal cells and neural precursor cells. Therefore, nestin is likely a marker of a poorly differentiated state in tumors.

In theory, some of the mechanisms by which poor differentiation could translate to clinical malignancy include a high proliferation rate, high proliferation to apoptosis ratio, infiltrativeness, incompleteness of surgical resection due to location of the tumor, older age of the patients at diagnosis, and resistance to adjuvant therapy. Since we corrected for the effect of age and incompleteness of resection by multivariate analysis, it seems unlikely that the association between nestin expression and survival is mediated through either of these variables. Nestin level had only a relatively weak effect on progression-free survival ($p<0.05$), suggesting that the strong effect of nestin on total survival is not mediated through resistance to adjuvant therapy. We did not see a significant correlation between MIB-1 proliferation index and nestin levels in A+OA II-III. In addition, MIB-1 proliferation index

did not reach significance as a prognostic factor in multivariate analysis. Thus, it seems unlikely that the effect of nestin on clinical malignancy is mediated through a direct effect on proliferation. This does not exclude the possibility that nestin could potentially increase net proliferation by inhibiting apoptosis. Evidence suggests that nestin does in fact inhibit apoptotic cell death in neural stem cells [27]. Additional studies are required to examine the possibility that nestin inhibits apoptosis in gliomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representative photomicrographs showing immunohistochemical staining for nestin in a glioma expressing low levels of nestin (glioma #1) and in a glioma expressing high levels of nestin (glioma #2). The image analysis method for evaluating nestin level is illustrated. The method consisted of measuring the area covered by the brown chromogen, corresponding to nestin, and the area covered by the blue counterstain, corresponding to tumor cell nuclei. The ratio of the area corresponding to nestin and the sum of the area corresponding to nestin and tumor cell nuclei was used as an estimate of nestin level per tumor cell.

Figure 2.

The level of nestin protein expression (median, 75th and 25th percentile) is different among grade II-III oligodendrogliomas (O II-III), grade II-III astrocytomas and oligoastrocytoma (A+OA II-III), and glioblastomas (GBM; grade IV astrocytomas) ($p < 0.0001$). Highest nestin expression is found in GBM, followed by A+OA II-III and O II-III.

Figure 3.

Kaplan-Meier survival curves showing total survival of A+OA II-III patients by nestin protein expression in the tumor tissue (UT Southwestern data). Nestin protein expression is inversely correlated with total survival.

Figure 4.

Kaplan-Meier survival curves showing total survival of A+OA III patients by nestin mRNA level (NCI Rembrandt data). Nestin mRNA expression is inversely correlated with total survival.

Table 1

Total survival multivariate analysis in the A+OA II-III group, Cox proportional-hazards regression.

Significant Variables	p	Relative Risk	95% Confidence Interval for Relative Risk
High nestin level	0.002	13.42	2.56 to 70.33
IDH 1/2 wt	0.004	6.99	1.91 to 25.66
Female gender	0.022	5.02	1.27 to 19.86
Subtotal resection	0.037	3.97	1.10 to 14.35

Nonsignificant variables: age, WHO grade (II vs. III), MIB-1 proliferation index, oligodendroglioma component (A vs. OA), type of adjuvant therapy (TMZ+rad vs. other)