Sox11 expression in astrocytic gliomas: correlation with nestin/c-Met/IDH1-R132H expression phenotypes, p-Stat-3 and survival

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Background: Sox11 is a transcription factor expressed in foetal and neoplastic brain tissue, including gliomas. It has been shown to suppress the tumourigenicity of glioma stem cells in vivo, thereby being hypothesised to function as a tumour suppressor.

Methods: We investigated the expression of Sox11 in 132 diffuse astrocytomas in relation to the regulator cell marker nestin, c-Met and IDH1-R132H, which have shown to be differentially expressed among the molecular subgroups of malignant gliomas, as well as to an inducer of astrocytic differentiation, that is, signal transducer and activator of transcription (p-STAT-3), clinicopathological features and survival.

Results: Sox11 immunoreactivity was identified in all tumours irrespective of grade, but being correlated with p-STAT-3. Three out of seven cases showed partial Sox11 promoter methylation. In > 50% of our cases neoplastic cells coexpressed Sox11 and nestin, a finding further confirmed in primary glioblastoma cell cultures. Furthermore, nestin, c-Met and IDH1-R132H expression differed among grade categories. Cluster analysis identified four groups of patients according to c-Met, nestin and IDH1-R132H expression. The c-Met/nestin high-expressor group displayed a higher Sox11 expression. Sox11 expression was an indicator of favourable prognosis in glioblastomas, which remained in multivariate analysis and validated in an independent set of 72 cases. The c-Met/nestin high-expressor group was marginally with shorter survival in univariate analysis.

Conclusions: We highlight the importance of Sox11 expression as a favourable prognosticator in glioblastomas. c-Met/nestin/IDH1-R132H expression phenotypes recapitulate the molecular subgroups of malignant glioma.

Glioblastoma (astrocytoma grade IV) ranks as the most common and most aggressive primary brain tumour in adults (Maher et al., 2001), its prognosis remaining uniformly fatal despite concerted efforts and advances in surgery, radiotherapy and chemotherapy (including temozolomide; Stupp et al., 2005). The low patient-survival rate and the lack of robust treatment options have propelled a search for markers, which could identify subgroups of patients likely to benefit from molecularly targeted therapies.

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The role of Sox11 in astrocytic gliomas

The tumour stem cell concept pertains to the existence of small populations of tumourigenic neoplastic cells with self-renewal capacity (Clarke et al, 2006). Glioma stem cells (GSCs) are remarkably similar to normal neural stem cells in terms of expression of neural stem/progenitor cell markers, and upon induction differentiate into neuronal or glial lineages (Chen et al, 2012). Glioma stem cells are notorious for their resistance to radio/chemotherapy owing to activation of DNA repair pathways mediated by CHK1/CHK2 (Bao et al, 2006). Although the most accredited stem cell marker is CD133, its reliability has been questioned by the observations that tumourigenicity is not restricted to CD133 + tumour compartment (Beier et al, 2007) and that CD133 –/nestin + isolated glioma cells are able to grow tumours in rodents (Wang et al, 2008). Nestin is an intermediate filament the expression of which in normal adult brain is restricted to the subventricular zone and the dentate gyrus where normal neural precursors reside (Dictus et al, 2007). However, nestin is not a definite marker of GSCs being also expressed by neural/glial progenitor cells (Chen et al, 2012). It is therefore best regarded as a marker of dedifferentiated state.

Sox11 belongs to a group of transcription factors within the high mobility group (HMG) box protein superfamily, characterised by high sequence homology within their DNA-binding HMG domain (Wegner, 1999). High mobility group serves two functions, namely DNA binding and partner selection, which allow the selective recruitment of Sox proteins to specific promoters and transcription factors (Wilson et al, 2002). Sox genes are divided into eight groups and their sequential expression regulates neurogenesis from the early to the late stages (reviewed in Bergslund et al, 2011). In particular, SoxG group (to which Sox11 belongs) is responsible for the induction and the early phases of neuronal gene expression, whereas SoxB1 (to which the stem cell marker Sox2 belongs) maintains the progenitor stage of neural cells, counteracting neuronal differentiation (reviewed in Bergslund et al, 2011). Sox11 is mainly involved in neural development and organogenesis during foetal life, being absent from most normal adult tissues (reviewed in Fenzo-Mendez, 2010). Recently, Sox11 has been advanced as a contributor to the pathogenesis of mantle cell lymphoma (Nygren et al, 2012), ovarian carcinoma (Brennan et al, 2009) and malignant gliomas (Weigle et al, 2005). Importantly, a link between GSCs and Sox11 is provided by a recent experimental study, documenting that GSCs are largely Sox11-negative and exogenous overexpression of Sox11 suppresses their tumourigenicity by inducing neuronal differentiation (Hide et al, 2009).

Microarray analyses have revealed subclasses of glioblastoma identified by congruence of genomic features that would otherwise be indistinguishable by morphology (reviewed in Chen et al, 2012). Although the number and types of subclasses vary depending on the sample pool and the methodology, the presence of proneural (PN) and mesenchymal (MES) subtypes has been confirmed by two independent studies (Phillips et al, 2006; Verhaak et al, 2010). These molecular subclasses differ with regard to survival rate (Colman et al, 2010; Vitucci et al, 2011), response to conventional therapies (Verhaak et al, 2010) and expression of stem and precursor cell markers, such as nestin (Colman et al, 2010). The MES subtype displays expression of MES markers such as MET (Phillips et al, 2006; Verhaak et al, 2010), whereas a major feature of the PN type are point mutations in isocitrate dehydrogenase (IDH)-1 gene, resulting in the expression of the mutant protein IDH1-R132H (Verhaak et al, 2010; Chen et al, 2012).

This study focuses on Sox11 expression in diffuse astrocytic tumours for which published information is limited to experimental data (Hide et al, 2009) and is organised in three parts. In the first part, we analysed the immunohistochemical expression of Sox11 in relation to standard clinicopathological parameters and survival using univariate analysis, and searched for Sox11 promoter methylation in the few cases with diminished or absent immunoreactivity. In the second part, we dealt with the relationships with nestin and the transcription factor, signal transducer and activator of transcription (p-STAT-3). The former was included because experimental data indicates that bona fide GSCs lack Sox11 while expressing nestin (Hide et al, 2009), but also because of its recently emphasised role in glioblastoma tumourigenesis (Lu et al, 2011). This relationship was investigated at the immunohistochemical but also at the flow cytometry level. The hallmark of glioblastoma p-STAT-3 (Brantley et al, 2008) promotes astrocytic differentiation (Rajan et al, 1998) and as such its expression may parallel that of Sox11. In the third part, we used three markers, namely nestin, c-Met and IDH1-R132H, which are differentially expressed among the molecular subclasses of glioblastoma (Verhaak et al, 2010) to segregate our cases into phenotypic subgroups analogous to the molecular subgroups. The ultimate goal was to evaluate whether Sox11 expression may vary according to the phenotypic subgroup of astrocytic tumours and may retain its prognostic utility in a multivariate analysis, including conventional prognosticators and the phenotypic subgroups. Finally, the prognostic effect of Sox11 was validated in an independent set of 72 glioblastoma patients.

MATERIALS AND METHODS

Patients’ description. This is a study of 132 adult patients with supratentorial diffuse infiltrating astrocytomas (grades II–IV) for whom archival primary tumour material at diagnosis, before radiotherapy, was available. Patients had been diagnosed in the First Department of Pathology, Laikon Hospital, National and KAPO University of Athens, and treated as well as followed-up in Evangelismos, Asklepieion and Metropolitan Hospitals between 1999 and 2008. In all cases, the diagnoses and grading were peer-reviewed according to the principles laid down in the latest World Health Organization (WHO) Classification (Louis et al, 2007). Informed consent was obtained from all patients and the study was approved by the University of Athens Medical School Ethics Committee. The demographic data of our patients are summarised in Table 1.

Immunohistochemical staining. Immunostaining was performed on paraffin-embedded 4 μm sections of formalin-fixed tumour tissue using the two-step peroxidase-conjugated polymer technique (DAKO Envision kit, DAKO, Carpinteria, CA, USA). The primary antibodies used are listed in Table 2.

Sox11 immunostaining was available in the entire cohort (132 cases), IDH1-R132H and nestin in 111 cases, c-Met staining in 92 cases and p-STAT-3 in 113 cases. Evaluation of IDH1-R132H, nestin, c-Met and p-STAT-3 staining was performed by one pathologist, without knowledge of the clinical information. Sox11 immunostaining was evaluated by using the computerised image analysis software Image Pro software v5.1 (Media Cybernetics Inc., Rockville, MD, USA) on a Pentium III PC (Intel, Hillsboro, OR, USA), which evaluated both expression and staining intensity. In each case, immunoreactivity was calculated in at least 500 tumour cells counted in several randomly chosen high power fields. As Sox11 is known to be absent in normal brain tissue, any level of nuclear immunoreactivity was recorded as positive. Validation of automated quantification was performed in 20 random cases in which Sox11 nuclear immunoreactivity was manually estimated. IDH1-R132H was recorded as positive when cytoplasmic staining of tumours was seen (Hartmann et al, 2010). Regarding c-Met expression, tumours were assigned into low expression (no immunoreactivity/low expression levels or staining intensity similar to that of normal brain tissue) or high expression group (moderate or strong immunoreactivity in >25% of tumour cells), as proposed by Kong et al (2009). Nestin intensity was in all
and Sox11 expression was performed. Cultures reached confluency, flow cytometric analyses for nestin were established, as previously described (Zisakis et al., 2010). The antibodies used are listed in Table 2. Isotype-specific antibodies were used as negative controls. Fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analysis of the data was performed using CellQuest software (BD Biosciences). The percentages of positive cells were calculated by the subtraction of the background from control stainings.

Methylation-sensitive high-resolution melting analysis sequencing. In order to detect the promoter methylation status of Sox11 gene, we used a real-time PCR approach, followed by high-resolution melting curve analysis. This is considered as a rapid, highly sensitive and efficient method displaying the sequence-dependent melting profile of an amplicon on a Light Cycler 480 (Roche Diagnostics, GmbH, Mannheim, Germany) in one single run. All sodium bisulphite-treated DNAs were analysed in triplicate. PCR products were also analyzed by Sanger sequencing to identify individual methylated CpGs, as previously described (Gustavsson et al., 2010).

Statistical analysis. Statistical analysis was performed by an MSc Biostatistician (GL). In the basic statistical analysis, Sox11 and p-STAT-3 were treated as continuous variables. c-Met and nestin expression were treated as dichotomous variables, that is, low or high, as already described. Associations of the molecules under study with clinicopathological parameters were tested using non-parametric tests, with correction for multiple comparisons (Kruskal-Wallis ANOVA, Mann-Whitney U-test, Fisher’s exact test and Spearman’s rank correlation coefficient, as appropriate).

Hierarchical cluster analysis was performed using the Ward method in order to classify our cases into subgroups on the basis of their differential expression of c-Met, nestin and IDH1-R132H. The dissimilarity between observations was evaluated using the Jaccard method.

Survival analysis was performed using death-by-disease as end point. The effect of various clinicopathological parameters (age, sex, tumour location, radiotherapy, chemotherapy, extent of surgical resection and histological grade) as well as Sox11, c-Met, nestin and IDH1-R132H immunoactivity on clinical outcome was assessed by plotting survival curves according to the Kaplan-Meier’s method and comparing groups using the log-rank test or the Wilcoxon Breslow test, as appropriate. p-STAT-3 was not included in survival analysis because its prognostic significance has been dealt with in a previous study of our group (Piperi et al., 2011). Numerical variables were categorised on the basis of cut-off values provided by ROC curves. Power estimation of the log-rank tests regarding Sox11 expression was performed using the Freedman method for estimation of censored data. Multivariate analysis was performed using Cox’s proportional hazard estimation model in order to evaluate the predictive power of each parameter independently of others. To avoid any ‘data-driven’ categorisation, numerical variables (age, Sox11 labelling index) were entered in multivariate analysis in continuous form.

Statistical calculations were performed using the statistical package STATA 11.0 for Windows (College station, TX, USA). Cluster analysis and subsequent figures were performed using R 2.12.0 for Windows (CRAN, Vienna, Austria). All results with a two-sided P level \(\leq 0.05\) were considered statistically significant.

Validation cohort. An independent set of patients with glioblastomas was used to validate the chosen cut-off values for the expression of Sox11 in univariate analysis. The results of univariate analysis was strong. Therefore, only the percentage of positive cells was taken into account for statistical analysis. The cut-off for high vs low nestin expression was 30%, as previously suggested (Rushing et al., 2010). These thresholds were evaluated using receiver operating characteristics (ROC) curves. For p-STAT-3 both the percentage of positive neoplastic nuclei and staining intensity was recorded, and a Histo-score (H-score) based on the percentage of stained neoplastic cells multiplied by staining intensity was calculated.

Human primary glioblastoma cell cultures. Three fresh glioblastoma samples were obtained in collection medium (Dulbecco’s modified Eagle’s medium/Ham’s F12, penicillin and streptomycin, amphotericin B), and primary glioblastoma cell cultures were established, as previously described (Zisakis et al., 2007). When cell cultures reached confluence, flow cytometric analyses for nestin and Sox11 expression was performed.

Flow cytometry analysis. At least 4 x 10⁶ cells were used for flow cytometric analysis. Cells were fixed in 1% paraformaldehyde (Sigma, St. Louis, MO, USA) for 10 min at room temperature. Fc receptors were blocked using 5% FBS (Invitrogen, Antisel SA, Greece) in PBS (Invitrogen). After washing, the cells were permeabilised in PBS supplemented with 0.5% FBS and 0.1% Triton-X-100 (Sigma). The antibodies used are listed in Table 2. Isotype-specific antibodies were used as negative controls. Fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analysis of the data was performed using CellQuest software (BD Biosciences). The percentages of positive cells were calculated by the subtraction of the background from control stainings.

Table 1. Demographic data of patients’ and validation cohort

| Variable          | Patients’ cohort (n = 132) Median (range) | Validation’s cohort (n = 72) Median (range) |
|-------------------|------------------------------------------|---------------------------------------------|
| Age               | 58 (19–84)                                | 60 (36–76)                                  |
|                   | Number of cases                           | Number of cases                              |
| Gender            |                                          |                                             |
| Male              | 80                                       | 44                                          |
| Female            | 52                                       | 28                                          |
| Location          |                                          |                                             |
| Frontal           | 26                                       | 18                                          |
| Temporal          | 25                                       | 11                                          |
| Parietal          | 31                                       | 12                                          |
| Occipital         | 9                                        | 1                                           |
| > 1 lobe          | 41                                       | 30                                          |
| Grade             |                                          |                                             |
| II                | 31                                       |                                              |
| III               | 20                                       |                                              |
| IV                | 81                                       | 72                                          |
| Events            |                                          |                                             |
| Death             | 77 (Follow-up: 1–41 months, median 11 months) | 68 (Follow-up: 1–24 months, median 10 months) |
| Censored          | 49 (Follow-up: 3–74 months, median 18 months) | 4 (Follow-up: 13–36, median 15.5 months)     |
| Surgery           |                                          |                                             |
| Partial           | 74                                       | 3                                           |
| Complete          | 48                                       | 68                                          |
| NA                | 10                                       | 1                                           |
| Radiotherapy      |                                          |                                             |
| Yes*              | 95                                       | 53                                          |
| No                | 17                                       | 3                                           |
| NA                | 20                                       | 16                                          |
| Chemotherapy (temozolomide) |                |                                             |
| Yes               | 39                                       | 54                                          |
| No                | 14                                       | 0                                           |
| NA                | 79                                       | 18                                          |

*Postoperative radiotherapy (a total dose of 60 Gy in 30–33 fractions).
survival analysis for Sox11 expression in the population group were used to calculate the required number of patients in the validation group for an adequately powered analysis (90%). In order to detect a hazard ratio (HR) of 0.348, as calculated in the patients’ group using a two-sided log-rank test, and to achieve 90% power at a 0.05 significance level, 58 patients would be needed. The validation group we used consisted of 72 patients, the demographic data of whom are shown in Table 1. These patients were diagnosed and treated at Red-Cross Hospital between 2007 and 2011.

## RESULTS

Sox11 expression in astrocytic tumours associations with clinicopathological features and analysis of Sox11 promoter methylation status. Sox11 immunoreactivity was nuclear and was observed in all cases in the population cohort and in 67 out of 72 cases of the validation cohort, ranging from 0.5% to 94%. Endothelial cells of tumoural vessels were also positive for Sox11, and therefore served as internal positive controls (Table 3; Figure 1). Normal brain did not display any Sox11 immunoreactivity, either in normal astrocytes or in endothelial cells. Although the staining intensity varied among tumours from moderate to strong, all the analyses regarding Sox11 immunoexpression were based on the percentage of positive cells, according to preliminary statistical analysis of our cohort, indicating that staining intensity did not correlate with any parameter in this investigation and the respective literature concerning other neoplasms (Ek et al, 2008; Brennan et al, 2009; Gustavsson et al, 2010; Nygren et al, 2012). Automatically and manually estimated labelling indices were strongly correlated ($p = 0.9743$) and directly comparable as the $P$ value of detecting a difference $> 5\%$ between the two measurements was $< 0.0001$ (Wilcoxon-matched paired one-sided test). Sox11 expression was not correlated with tumour histological grade (Kruskal-Wallis ANOVA, $P > 0.10$, Figure 2A) or any other clinicopathological features.

Seven samples displaying different levels of Sox11 expression (ranging between 0 and 95%) were analysed for Sox11 promoter methylation status. Cases showing reduced ($< 40\%$) or absent Sox11 immunoexpression displayed at least partially methylated promoter sequences, whereas those having enhanced Sox11 expression ($> 60\%$) displayed unmethylated promoters (Figure 3).

Nestin, c-Met, IDH1-R132H expression in astrocytic tumours and associations with clinicopathological features. Nestin and c-Met expression was cytoplasmic and observed in 85.6% (95/111)

### Table 2. Characteristics of primary antibodies used in IHC and FACS

| Protein   | Clone     | Company               | Catalogue no. | Raised in | Positive controls | No of stained slides for IHC | Antigen retrieval method for IHC | Dilution and incubation time for IHC | Dilution and incubation time for FACS |
|-----------|-----------|-----------------------|---------------|-----------|-------------------|-------------------------------|-----------------------------------|--------------------------------------|--------------------------------------|
| SOX11     | Polyclonal| Spring, Pleasanton, CA, USA | 110401A       | Rabbit    | Mantle cell lymphoma | 132                          | Citrate buffer, pH 9          | 1:1000, 1 h (room temperature) | 1:100, 16 h (4°C)                     |
| c-Met     | Polyclonal| Invitrogen, Greece    | 182257        | Rabbit    | Breast carcinoma   | 111                          | Citrate buffer, pH 6          | 1:200, 18 h (4°C)                  |                                      |
| Nestin    | Monoclonal| Santa Cruz, CA, USA   | Sc-23927      | Mouse     | Kidney             | 92                           | Citrate buffer, pH 6          | 1:100, 1 h (room temperature)    | 1:50, 16 h (4°C)                     |
| IDH1-R132H| monoclonal| Dianova, Hamburg, Germany | DIA H09      | Mouse     | Oligodendroglioma, diffuse astrocytoma | 111 | Citrate buffer, pH 6 | 1:50, 18 h (4°C) |                                      |
| p-STAT-3  | monoclonal| Cell Signaling, Danvers, MA, USA | D3A7 XP   | Rabbit    | Breast carcinoma   | 113                          | Citrate buffer, pH 6          | 1:50, 18 h (4°C)                  |                                      |

Abbreviations: FACS = flow cytometric analysis; FITC = fluorescein isothiocyanate; PE = phycoerythrin; p-STAT-3 = signal transducer and activator of transcription.

*Incubation with secondary antibody donkey anti-rabbit-PE (558416, BD Biosciences) at dilution 1:200 for 1 h at room temperature.

*Incubation with secondary antibody goat anti-mouse-FITC (sc-2010, Santa Cruz Biotechnology) at dilution 1:200 for 1 h at room temperature.

### Table 3. Distribution of SOX11, p-STAT-3, c-Met, nestin and IDH1-R132H expression according to histological grade

| Histological grade | SOX11 expression (%) | p-STAT-3 H-score | Nestin expression | IDH1-R132H expression |
|--------------------|-----------------------|------------------|-------------------|-----------------------|
|                    | Median (range)        | p-STAT-3 H-score | Low n | High n | <30% n | ≥30% n | Absent n | Present n |
| II                 | 41.5 (1.3–94)         | 23 (0–180)       | 14   | 1     | 14     | 8      | 19      | 8         |
| III                | 44.5 (9–76)           | 10 (0–140)       | 11   | 1     | 12     | 4      | 10      | 5         |
| IV                 | 44 (7–85)             | 10 (0–225)       | 46   | 18    | 25     | 46     | 55      | 16        |

Abbreviations: H-score = Histo-score; p-STAT-3 = signal transducer and activator of transcription.
and 46.7% (43/92) of the examined cases, respectively, (Table 3). The IDH1-R132H immunostaining was cytoplasmic and was evaluated as positive or negative, as described previously (Hartmann et al, 2010). None of the IDH1-R132H-positive cases displayed high c-Met expression (Fisher’s exact test, $P = 0.0030$). Accordingly, all but three cases positive for IDH1-R132H displayed a lower nestin expression ($< 30\%$; Fisher’s exact test, $P < 0.0001$).

c-Met and nestin expression were higher in glioblastomas when compared with diffuse and anaplastic astrocytomas (Fisher’s exact test, $P = 0.030$ and $P = 0.001$, respectively), whereas IDH1-R132H immunoreactivity was mostly seen in grades II/III (Fisher’s exact test, $P = 0.0250$). All other associations with clinicopathological features were not significant ($P > 0.10$).

Associations between Sox11 and nestin, c-Met, IDH1-R132H or p-STAT-3 expression. Sox11 expression was positively correlated with p-STAT-3 H-score and nestin expression ($\rho = 0.3705$, $P = 0.0001$ and $\rho = 0.3029$, $P = 0.012$, respectively). Interestingly, in 58.55% (65 out of 111) of the examined cases, the sum of Sox11 and nestin labelling indices exceeded 100% by a median of 29% (range 2%–72%), clearly denoting coexpression of the two molecules by at least a subset of neoplastic cells. Two such cases with significant coexpression are shown in Figure 1. No significant relationship between Sox11 and c-Met or IDH1-R132H was documented ($P > 0.10$).

Figure 1. (A, B) Immunohistochemical expression of Sox11 in a grade-II diffuse astrocytoma (A) and a grade-IV (B) glioblastoma. (C, D) Immunohistochemical expression of Sox11 (C) and nestin (D) at the same region in a glioblastoma case. Both proteins were abundantly expressed by the majority of the neoplastic cells. (E, F) High Sox11 (E) and moderate nestin. Nestin (F) expression at the same region in a diffuse astrocytoma case. Many of the cells expressing Sox11 were also positive for nestin.

Figure 2. Box-plots illustrating the distribution of Sox11 expression among histological grade (A) and the subgroups that emerged from cluster analysis in the entire cohort (B).

Figure 2. Box-plots illustrating the distribution of Sox11 expression among histological grade (A) and the subgroups that emerged from cluster analysis in the entire cohort (B).
Coexpression of Sox11 and nestin in a subset of primary glioma cells. Flow cytometric analysis was used to verify Sox11 and nestin’s coexpression in three primary glioblastoma cell cultures. Representative data of single and double staining analysis for Sox11 and nestin are shown in Figure 4. Single staining revealed that both proteins are independently expressed in primary glioblastoma cells, with nestin expression separating the culture into two distinct populations. Furthermore, double staining revealed a subset population of 9.14% of gated glioma cells to be positive for both Sox11 and nestin (Figure 4).

Classification of cases into subgroups according to nestin, c-Met and IDH1-R132H expression. Hierarchical cluster analysis was performed using IDH1-R132H, c-Met and nestin expression. The dendrogram of this analysis is presented in Figure 5. According to this dendrogram, four groups consisting of cases with similar expression patterns were identified. The first group included 17 IDH1-R132H-negative cases overexpressing c-Met, 76.5% (13 out of 17) of which displayed also high levels of nestin. All cases in the second group (18 patients) overexpressing nestin were negative for IDH1-R132H and displayed low levels of c-Met expression. Accordingly, all cases in the third group (18 patients) were positive for IDH1-R132H and displayed low levels of nestin and c-Met expression. The remaining 17 patients of the fourth group neither overexpressed c-Met/nestin nor expressed IDH1-R132H. When this analysis was repeated in glioblastomas, the same four subgroups emerged.

Interestingly, Sox11 expression was marginally higher in the first two subgroups characterised by the presence of c-Met and/or nestin overexpression (Mann-Whitney U test, $P=0.0548$, Figure 2B). Although Sox11 expression seemed to be higher in the first subgroup characterised by c-Met overexpression than in the third group represented by IDH1-R132H-positive cases, this difference failed to attain statistical significance ($P>0.10$).

Survival analysis. Univariate survival analysis was carried out in grades II/III and in glioblastomas separately. The results are presented in Table 4. Sox11 overexpression was correlated with improved overall survival in glioblastomas ($P=0.0002$, Figure 6A), irrespective of or despite its coexpression with nestin (log-rank test, 

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cases displaying IDH1-R132H positivity (Wilcoxon Breslow test, \(P = 0.0736\), Figure 6C), which, however, was not reproduced when grades II/III and IV were analysed separately (\(P > 0.10\)). Interestingly, there was an almost significant decreased probability of survival in the first two subgroups of patients characterised by c-Met and/or nestin overexpression in grades II/III (\(P = 0.0505\)). The worse behaviour of these expression subgroups is illustrated in Figure 6D.

Multivariate survival analysis, including all parameters for the patients for whom staining results for the molecules under study were available, is presented in Table 5. Sox11 expression emerged as an independent prognosticator (HR = 0.975, \(P = 0.009\)), along with histological grade, patients’ age and the administration of radiotherapy. Importantly, Sox11 expression prognostic significance remained when analysis was restricted to glioblastomas (HR = 0.339, \(P = 0.010\)).

Survival analysis-validation group. The overall survival was significantly lower in the Sox11 low-expressor group compared with the Sox11 high-expressor group (log-rank test, \(P < 0.0001\), Figure 6B). This effect remained after adjustment for patient’s age and treatment (HR = 0.27, \(P < 0.0001\)), thus corroborating the respective findings established in the patients’ cohort.

**DISCUSSION**

Since their discovery in 2006, GSCs have been advanced as the primary ‘culprit’ in the inherent resistance of glioblastoma to chemo/radiotherapy and the invariable maintenance of residual disease after conventional therapies (Kim et al, 2011). Although the tumour stem cell hypothesis has been challenged because of the lack of reliable stem cell markers, it has certainly revolutionised our understanding of antineoplastic therapy. Eliminating this minority cell population either by blocking components of pathways or ‘stemness’ factors that sustain its growth, or by upregulating the expression of factors that promote differentiation might prove a more biologically rational and efficient approach for the eradication of tumours, such as glioblastoma, resistant to standard therapies (Kim et al, 2011).

The vast majority of our diffuse astroglial tumours exhibited Sox11 immunoreactivity. The pattern of staining was nuclear, as reported by other investigators in foetal brain tissue (Haslinger et al, 2009), mantle cell lymphoma (Nordström et al, 2012), ovarian carcinoma (Brennan et al, 2009) and medulloblastoma (de Bont et al, 2008), conforming to the nuclear localisation signals conserved among all Sox proteins. Indeed, the lack of nuclear staining reportedly correlates with the absence of Sox11 mRNA in normal and neoplastic tissues (Ek et al, 2008). The abundant expression of Sox11 in our cases is in harmony with the results of primary glioblastoma cell cultures and those obtained by quantitative real-time PCR revealing a 5- to >600-fold Sox11 mRNA overexpression in malignant gliomas (Weigle et al, 2005). The absence of Sox11 immunoreactivity in adjacent normal brain tissue along with its rather uniform distribution of its levels among low- and high- grade cases lends support to the assumption that, following downregulation in normal adult brain, Sox11 is reactivated early in gliomagenesis, as a result of dedifferentiation (Weigle et al, 2005). In keeping with this interpretation, de Bont et al (2008) noticed more prominent staining in medulloblastoma than that in ependymoma. Therefore, Sox11 might also be of potential value as a diagnostic marker of neoplastic astrocytes. A similar absence of correlation between Sox11 expression and histological grade has been noted in ovarian carcinoma (Sernbo et al, 2011), as opposed to meningiomas where marked upregulation of Sox11 mRNA has been observed in grade III (Stuart et al, 2011).
The role of Sox11 in astrocytic gliomas

Despite the upregulation of Sox11 mRNA in some tumours as alluded to, the molecular mechanisms by which Sox11 modulates oncogenesis are largely unknown and somewhat controversial (Penzo-Mendez, 2010). It has been hypothesised that Sox11 may contribute to the pathogenesis of mantle cell lymphoma by regulating genes involved in cell proliferation and apoptosis (Klein and Assoian 2008). Also, in a neuroblastoma cell line, knockdown of Sox11 augmented the expression of the proapoptotic gene BNIP3 and decreased the expression of the antiapoptotic gene TANK (Jankowski et al, 2006). It is of interest that Sox11, which shares considerable sequence homology to Sox11, harbours both proapoptotic and antiapoptotic properties, its function being tissue specific and dependent on external signals (Penzo-Mendez, 2010).

Perhaps of even greater clinical relevance is our observation that increased levels of Sox11 nuclear expression were associated with a more favourable clinical outcome in both univariate and multivariate analysis. To the best of our knowledge, this is the first attempt to delineate the potential utility of Sox11, harbours both proapoptotic and antiapoptotic properties, its function being tissue specific and dependent on external signals (Penzo-Mendez, 2010).

Table 5. Cox’s proportional hazards model including all molecules under study in the entire cohort (n=72 patients, model A), as well as in glioblastomas (n=51, model B)

|                | HR       | P-value | 95% CI of HR |
|----------------|----------|---------|--------------|
| A              |          |         |              |
| Sox11 expression | 0.975    | 0.009   | 0.957 0.994  |
| Histological grade | 1.952    | 0.016   | 1.135 3.358  |
| Patients’ age   | 1.037    | 0.002   | 1.013 1.062  |
| Expression subgroup | 1.032    | 0.922   | 0.552 1.927  |
| Administration of radiotherapy | 0.369    | 0.013   | 0.169 0.809  |
| Surgery (Partial vs complete) | 0.870    | 0.689   | 0.441 1.717  |
| B              |          |         |              |
| Sox11 expression | 0.339    | 0.010   | 0.149 0.771  |
| Patients’ age   | 1.043    | 0.018   | 1.007 1.080  |
| Expression subgroup | 1.002    | 0.836   | 0.987 1.017  |
| Administration of radiotherapy | 0.373    | 0.043   | 0.144 0.967  |
| Surgery (Partial vs complete) | 1.042    | 0.917   | 0.481 2.258  |

Abbreviations: CI = confidence interval; HR = hazard ratio. Bold entries indicate statistical significance.

Figure 6. Kaplan-Meier survival curves according to Sox11 (A, B) expression in glioblastomas in patient’s (A, P=0.0002) and in validation cohort (B, P<0.0001), IDH1-R132H positivity (C, P=0.0832) in the entire cohort, as well as the expression subgroups, which emerged from cluster analysis (D, P=0.0505).
behaviour in vivo (Conrotto et al., 2011). Fourth, experiments in the induced mouse glioma cell line NSCL61 have disclosed that overexpression of Sox11 blocked their tumourigenicity and recurrent glioblastomas, which are enriched in GSCs were Sox11-negative. In addition, downregulation of Sox11 mRNA resulted in diminished patient survival (Hide et al., 2009). Interestingly, Sox4 is also a favourable prognostic marker in bladder cancer (Aaboee et al., 2006) and medulloblastoma (de Bont et al., 2008).

A significant positive relationship was documented between Sox11 and p-STAT-3 expression. The signal transducer and activator of transcription (p-STAT-3) is known to promote neurite growth (Moore et al., 2011) and induces astrocytic differentiation during central nervous system (CNS) development (Rajan et al., 1998). Therefore, the relationship between these two transcription factors may reflect their functional similarity. It is worthy of note that silencing of Sox11 in mantle-cell lymphoma cell lines caused a dysregulation of STAT-1 transcription (Conrotto et al., 2011). Whether a similar interaction may exist between Sox11 and STAT-3 is presently unknown.

An intriguing finding emerging from the present investigation is the coexpression of nestin and Sox11 by a subset of neoplastic cells (as high as 72%) in almost 60% of our cases, and corroborated by flow cytometry in three primary glioblastoma cell cultures. The percentage may be even higher as the presence of a few cells double positive for both nestin and Sox11 in the remaining cases cannot be ruled out. Given that the former is a marker of undifferentiated cells, whereas the latter a transcription factor promoting differentiation of neural precursors, this finding seems paradoxical and at variance with the reported inhibition of nestin, following forced upregulation of Sox11 in the induced mouse glioma cell line NSCL61 (Hide et al., 2009). At present, it is not clear whether these double-positive nestin and Sox11 cells are GSCs or glial precursors. However, immunohistochemistry in embryonic spinal cord sections has clearly shown that the two proteins are not mutually exclusive as there exist two populations of nestin-expressing cells in foetal CNS, that is, those expressing Sox1B1 and those expressing Sox11 (Tanaka et al., 2004). Our findings may be explained by the fact that Nestin gene expression in the early neural tube and possibly in neural primordial cells, is driven by the synergic interaction between group B1/C Sox and Class III POU (Pit-Oct-Unc) transcription factors, binding sites for which are found on interaction between group B1/C Sox and Class III POU (Pit-Oct-Unc) transcription factors, binding sites for which are found on

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