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RESEARCH ARTICLE

Identification of *OLIG2* as the most specific glioblastoma stem cell marker starting from comparative analysis of data from similar DNA chip microarray platforms

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Abstract Despite advances in surgical and adjuvant treatments, overall survival of glioblastoma (GBM) patients remains poor. The cancer stem cell concept suggests that a rare stem cell population, called glioma stem cells (GSCs), has high ability to self-renewal leading to recurrence in GBM. The identification of specific markers of GSCs would provide a powerful tool to detect and to characterise them in order to develop targeted therapies. We carried out a comparative analysis based on the identification of inter-study concordances to identify the genes that exhibit at best differential levels of expression between GSC-enriched cell cultures and differentiated tumour cell cultures from independent studies using DNA chip microarray technologies. We finally studied the protein expression of the marker we considered the most specific by immunohistochemistry and semi-quantitative analysis on a retrospective series of 18 GBMs. Of the selected studies, 32 genes were retained. Among them, eight genes were identified to be overexpressed in GSC-enriched cultures compared to differentiated tumour cell cultures. Finally, among the eight genes, oligodendrocyte lineage transcription factor 2 (*OLIG2*) was characterised by the most different expression level in the "GSC model" compared to the "differentiated tumour cells model". Our approach suggests that *OLIG2* is the most specific GSC marker; additional investigations with careful considerations about methodology and strategies of validation are, however, mandatory.

Keywords Olig2 · Glioblastoma · Glioma stem cells · Microarray studies

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Introduction

Glioblastoma (GBM) (World Health Organization grade IV glioma) is the most common primary brain tumour and the most lethal among gliomas in adults [1]. Despite advances in surgical and radiochemotherapeutic treatments, the prognosis of GBM patients remains poor with an average survival of only 12–18 months [2].

Tumour regrowth implies that GBM includes a population of cells that are resistant to therapy and maintain the ability to proliferate. A population of cells with stem-like cell properties has been identified in brain tumours [3–5], and several research teams have reported that these cells are relatively resistant to chemotherapy and radiation [6–8]. Furthermore, these cells could partially explain the known cellular heterogeneity of GBM [9].

The stem cell hypothesis may affect the way in which tumours are diagnosed and treated. The therapeutic aim would change from eliminating the bulk of rapidly dividing, but terminally differentiated components of the tumour, to refocusing on the stem cell population that fuels tumour growth [10, 11]. Such a transition highlights the need for laboratory work to identify true signatures of "stemness". Such studies require pure populations of cells; this is especially true for cancer stem cells that are expected to be rare. Consequently, their expression signatures would be swamped by the majority of non-stem cells in whole tumour samples, resulting in an average signature for the mixed population, rather than a specific signature for cancer stem cells [10].

A standard in vitro method called "neurosphere assay" has been proposed to enrich cancer stem cells, also called tumour-initiating cells (TICs), from brain tumours [12]. In fact, the neurosphere assay was one of the original methods used to identify putative cancer stem cells within brain tumours [3]. Neurosphere assays are carried out in selective neurobasal serum-free media supplemented with growth factors, in which glioblastoma stem cells (GSCs) (name of cancer stem cells or TICs used for glioma) as well as normal neural stem cells are able to continually divide and form multipotent clonal spheres called neurospheres. In contrast, the more differentiated cells, incapable of self-renewal and multipotency, die off with serial passages. GBM-derived neurosphere cultures are heterogeneous cell clusters that consist of stem cells, various progenitor cells and more differentiated cells. Despite the fact that these assays are currently used as the standard in vitro method for identifying the presence of GSCs, there are some limitations and the reliability is still under debate [12, 13]. Indeed, the population of cells isolated by this method represents a heterogeneous rather than a uniform population of cells that are in fact moderately enriched for GSCs [12]. Nonetheless, neurosphere cultures remain informative and important surrogates especially to show a self-renewal phenotype for GSCs versus non-GSCs. Furthermore, neurosphere cultures have the ability to be tumorigenic in vivo and to form subsequent heterogeneous tumours resembling human GBM in orthotopic xenograft models. Nowadays, serial transplantation assays into mice brains seem the best way to enrich tumours for stem cells; unfortunately, this method remains a heavy and time-consuming procedure.

Another method concerns the isolation of GSC subpopulations by the use of flow cytometry technology based on cell surface antigen expression [14]. However, these procedures for the enrichment/isolation of GSCs remain still imperfect and require improvements, as a consequence of the lack of universal markers [13].

The purpose of this work was to identify markers potentially specific for GSCs by performing a comparative analysis of data from similar DNA chip microarray platforms to select gene candidates that are differentially expressed between differentiated adherent tumour cells and cultures enriched in GSCs. We next addressed the expression level of the gene candidate supposed to be the best GSC marker using immunohistochemistry, a daily practice method used in surgical pathology laboratories.

Materials and methods

Selection of published studies for analysis

To identify which studies were eligible for inclusion in our comparative analysis, we performed a review of the literature from January 2000 to February 2013 by means of the "National Library of Medicine's PubMed", using different combinations of the following keywords: "glioblastoma", "glioma", "gene microarray", "microarray", "stem cell", "neurosphere", "spheroid", "primary", "tumour" and "tumour initiating". The inclusion criteria were as follows:

- Study of primary culture of human GBM
- GSC-enriched cell culture and differentiated tumour cell culture in the same study
- Comparison of genomic expression by DNA chip microarray

Microarray studies performed on commercially available GBM cell lines were excluded because these cell lines might not mirror the phenotype and genotype of human GBM [15].

This led to the selection of only three studies published by Lee et al. in 2006 [15], Schulte et al. in 2011 [16] and De Rosa et al. in 2012 [17].

Method of analysis

The data reported in the three publications were compared with the aim to coherently identify the most differentially expressed genes between the "GSC model" and the "differentiated tumour cells model".

Our gene selection criteria were defined as follows. The selected genes were differentially expressed in at least two separate studies where the differential expressions were consistent. A minimum variation in gene expression levels was defined on the basis of the binary logarithm of the ratio (r) between expression levels in the GSC model and the differentiated tumour cells model with a threshold of $\log_2 r<-2$ or >2.

The method used in this work is similar (but not identical) to the method used in a study of Rorive et al. [18].

Description of the three selected studies

Table 1 describes the mean characteristics of the three selected studies.

The biological materials used included both serum-free cultures of glioblastoma tumour cells for growth of neurospheres (GSC model) and classical cultures with serum-enriched media (differentiated tumour cells model). Only one study [15] was added for comparison cultures enriched in GSCs obtained from a mouse orthotopic xenograft model (data not shown). Moreover, one study [16] studied two types of glioblastoma stem-like cell (GS) lines: a cell line which displayed a full stem-like phenotype (GSf) and a proneural gene expression signature, and a cell line which displayed a restricted stem-like phenotype (GSr) and a mesenchymal gene expression signature.

As shown in Table 1, the three selected studies used the same DNA microarray Affymetrix Platform HG-U133.2.0. Consequently, the same set of genes was analysed and their expression levels are comparable between them.

Table 1 also shows the number of genes (from 20 to more than 1000) in each of the three studies for which the expression level is available and usable for our comparative analysis. It should be noted that Lee et al. [15] used two different probe sets: a NBE-specific probe sets and a NSC-enriched probe sets. The latter was drawn from three prior different studies [19–21] corresponding to a set highly enriched for stem cell-related genes. In our study, we considered the two probe sets for our comparative analysis.

To be able to include in our analysis the results provided by Schulte et al. [16], we calculated the log-ratio values from the raw data of gene microarray analysis available on the National Library of Medicine's PubMed site through the GEO DataSets access number referred by the authors (GSE23806).

Expression of selected markers in normal adult brain

We performed a search of the protein expression of the selected markers in the human adult normal brain. For this, we used the immunohistochemical data available in March 2013 on the online search engine "The Human Protein Atlas". The Human Protein Atlas provides protein expression profiles based on immunohistochemistry for a large number of human tissues and cancers assembled in tissue microarrays. The generated tissue microarrays include samples of normal brain tissue including cerebral cortex, hippocampus, lateral ventricle and cerebellum samples. The immunohistochemically stained tissues were annotated for the different tissue-specific cell types. Basic annotation parameters include an evaluation of staining intensity (negative, low, moderate or strong) and an evaluation of percentage of stained cells (0, <25, 25–50, >75 %) [22–24, www.proteinatlas.org].

Retrospective clinical series

We analysed the protein product of our gene candidate in a small retrospective series of 18 GBMs. For two of them, adjacent normal brain was also available and submitted to this analysis.

Given the great heterogeneity of GBMs, we decided to include nine primary (isocitrate dehydrogenase 1 (IDH1) non-mutated) and nine secondary (IDH1 mutated) GBMs. The presence of IDH1 mutation was determined by polymerase chain reaction (PCR) which is a daily practice method in our lab. Furthermore, we performed immunohistochemistry to display IDH1 mutation using a specific antibody anti-human IHD1 R132H provided by Dianova (Dianova GmbH, Germany) (DIA-H09, dilution 1/200).

The patients underwent a subtotal or macroscopically complete tumour resection at the Erasme University Hospital (Brussels, Belgium) between July 2011 and March 2013,

Table 1 Mean characteristics of the three selected studies

Reference	Number of cultures		DNA microarray platform	Methods of GBM stem cell isolation for DNA microarray analysis	Number of genes with available expression
	GSC model	Differentiated tumour cells model			
17	6	6	Affymetrix HG-U133 2.0	Specific culture media for growth of neurospheres (DMEM media without serum with B-27, penicillin/streptomycin, heparin, EGF and bFGF)	29
16	27	4	Affymetrix HG-U133 2.0	Specific culture media for growth of neurospheres (Neurobasal media with B-27/vitamin A, glutamine, penicillin/streptomycin, heparin, EGF, bFGF, NSF-1 and LIF)	>1000
15	20	20	Affymetrix HG-U133 2.0	Specific culture media for growth of neurospheres (Neurobasal media with B-27/N2, EGF and bFGF) SCID mouse, orthotopic xenografts	63[a]

[a] From two different probe sets: NBE-specific probe sets and NSC-enriched probe sets. The latter was drawn from three prior different studies [19–21]

and all tissue samples analysed in this study came from the archives of the Department of Pathology of the Erasme University Hospital. All patients gave informed consent before entering in the study. The clinical and biological data recorded for each patient are summarised in Table 2.

Immunohistochemistry and semi-quantitative analysis

Standard immunohistochemistry was applied to 5-μm thick sections to display Olig2 expression using a specific antibody provided by Chemicon-Millipore (Temecula, CA, USA) (ab9610, dilution 1:500) and was performed on the BOND-MAX. Briefly, as previously described [25], the immunohistochemical expression was visualised by means of streptavidin-biotin-peroxidase complex kit reagents (BioGenex, San Ramon, CA, USA) with diaminobenzidine/H_2O_2 as chromogenic substrate. Finally, the sections were counterstained with haematoxylin.

Semi-quantitative analysis was performed by two observers (ALT and CB). The staining was assessed by means of two features: staining intensity (absent, low, moderate or strong) and labelling index (0, no staining; +, <5 % positive cells; ++, 5–25 % positive cells; +++, >25 % positive cells).

Results

Concordance analysis of gene expression

From the three selected studies based on similar DNA chip microarray (Affymetrix Platform HG-U133 2.0) described above, 32 genes, whose expression data reported in at least two different studies were consistent, were retained based on the log-ratio selection criterion. These genes are presented in Supplementary Table 1.

Among them, eight genes (oligodendrocyte lineage transcription factor 2 (*OLIG2*), protein tyrosine phosphatase receptor type Z polypeptide 1 (*PTPRZ1*), inhibitor of DNA binding 4 (*ID4*), *OLIG1*, cyclin D2 (*CCND2*), aquaporin 4 (*AQP4*), neurocan (*NCAN*) and sex determining region Y-box 2 (*SOX2*)) are the most strongly overexpressed in cultures enriched in GSCs (GSC model) compared to serum-cultured GBM cells (differentiated tumour cells model). Analysing the eight gene data, we noted that there are three genes (*OLIG2*, *PTPRZ1* and *ID4*) whose differential expression is common across the three studies; the five others are genes for which differential expression levels are concordant across two studies and exhibit the highest log-ratios. Their main functions (see Table 3) show that all these genes are involved in the central nervous system development. Four of them are involved in the maintainance of undifferentiated status of normal stem cells (*OLIG2*, *SOX2*, *PTPRZ1* and *NCAN*), three in cell adhesion (*PTPRZ1*, *NCAN* and *AQP4*) and one in cell cycle regulation (*CCND2*).

Table 2 Major characteristics of the clinical retrospective series used for immunohistochemical analysis of the expression of the selected marker

Clinicopathological characteristics Maximal follow-up: 27 months	Glioblastomas (GBMs) (n=18)
Primary GBM	9
Secondary GBM[a]	9
Recurrence	4
Age (years)/median (range)	
Primary GBM	63 (47–77)
Secondary GBM[a]	44 (34–50)
Sex	
Male	7
Female	11
Surgery (macroscopically)	
Complete	8
Partial	10
Treatment before surgery of GBM[b]	
Radiotherapy	3
Radiotherapy + TMZ	4
None	2
Adjuvant treatment (after surgery of GBM)	
Primary GBM	
Radiotherapy + TMZ	9
Secondary GBM	
Chemotherapy (TMZ or others)	2
Bevazicumab (avastin)	1
Radiotherapy + TMZ	2
Bevazicumab + chemotherapy	4
EGFR amplification	
Present	10
Absent	6
Unknown	2
Methyl-guanine methyltransferase (MGMT) promoter methylation	
Present	13
Absent	5
Death from glioblastoma	7

[a] Classification in secondary glioblastomas based on the presence of preexisting histological lesions and/or presence of IDH1 mutation

[b] Concerning secondary GBM

Expression of our eight gene candidates in normal adult brain

As we wanted to identify a marker potentially specific for GSC, we studied the expression of our eight gene candidates in human adult normal brain. This analysis aimed to select among the eight gene candidates, the marker with the lowest expression in adult normal brain and, above all, which seems

Table 3 Main functions and expression ratios for our eight gene candidates identified as the most differentially expressed

Gene	Official denomination	Main functions	Log_2 expression ratio	References
OLIG2	Oligodendrocyte lineage transcription factor 2	CNS development	5.59 (n=40)	[15]
		Differentiation in oligodendrocytes	8.75 (n=31)	[16][a]
			>3.33 (n=12)	[17][b]
PTPRZ1	Protein tyrosine phosphatase receptor type Z polypeptide 1	CNS development	3.56 (n=40)	[15]
		Cell adhesion	8.78 (n=31)	[16][a]
		Migration	>3.33 (n=12)	[17][b]
ID4	Inhibitor of DNA binding 4	CNS development	2.87 (n=40)	[15]
		Astrocytic and neuronal differentiation	4.69 (n=31)	[16][a]
			>3.33 (n=12)	[17][b]
OLIG1	Oligodendrocyte lineage transcription factor 1	Differentiation in oligodendrocytes	5.63 (n=40)	[15]
		Myelin repair	9.98 (n=31)	[16][a]
CCND2	Cyclin D2	Cycle cell regulation	5.98 (n=40)	[15]
		Neurogenesis	6.76 (n=31)	[16][a]
AQP4	Aquaporin 4	Solute transport (K^+ and Cl^-)	6.32 (n=40)	[15]
		CNS development	6.10 (n=31)	[16][a]
NCAN	Neurocan	Adhesion	4.48 (n=40)	[15]
		CNS development	7.75 (n=31)	[16][a]
SOX2	Sex determining region Y-box 2	CNS development	3.45 (n=40)	[15]
			8.03 (n=31)	[16][a]

Expression ratio: ratio between expression in the "GSC model" in vitro and the "differentiated tumour cells model" (n=number of samples)

[a] Expression ratios calculated from the raw data (GEO DataSets access number: GSE23806)

[b] Expression ratios could be significantly higher than mentioned (no access to raw data available)

the most specific for glial lineage (thus ideally with no expression in neurons and neuropil). Table 4 shows the protein expression levels of these markers as published in the online database The Human Protein Atlas. These data highlight a selective expression of Olig2 in a subset of glial cells while the other markers show an expression in neurons and/or in neuropil, suggesting they are less specific for glial cells.

Semi-quantitative analysis of Olig2 protein expression

The previous data confirm Olig2 as the best candidate for GSC marker. We indeed observed that Olig2 is characterised by the most different expression level in the GSC model compared to the differentiated tumour cells model. Moreover, we noted a selective expression of Olig2 in a subset of glial cells in adult normal brain both in The Human Protein Atlas and in our immunohistochemical analysis of adjacent peritumoural normal brain (Fig. 1a).

Next, we studied Olig2 protein expression on a series of GBMs by immunohistochemistry to assess its application as a potential GSC marker using a simple method used in routine.

In GBM, expression of Olig2 was predominantly nuclear and cytoplasmic in rare cases. All cases were positive. The staining intensity was moderate to strong in all tumours. The labelling index was variable: + (<5 %) in 7/18 cases (Fig. 1b), ++ (5–25 %) in 4/18 cases (Fig. 1c) and +++ (>25 %) in 7/18 cases (Fig. 1d).

Using this small series, we studied whether Olig2 is associated with clinical or biological characteristics. Table 5 summarises the distribution of the Olig2 labelling scores according to the main histological and biological characteristics of our clinical series; test of significance was not performed because of the small number of cases. The percentage of tumour cells positive for Olig2 tended to be higher in secondary glioblastomas (+++ in 5/9 of cases) than in primary glioblastomas (+ in 5/9 cases). *Methyl-guanine methyltransferase (MGMT)* promoter methylation was often associated with a large number of Olig2-positive tumour cells (+++ in 6/13 of cases). Our clinical series was too recent to make a pertinent analysis of recurrences. However, the expression of Olig2 was scored +++ in two of the four recurrences. No trend of predominant expression of Olig2 appeared depending on the age or the presence/absence of *EGFR* gene amplification.

Discussion

The identification of markers specific for GSCs would provide not only a powerful tool to detect and to isolate them from the tumour but also to develop therapies targeted to these cells.

Table 4 Immunohistochemical protein expression levels of the eight gene candidates in human adult normal brain

Protein	Expression in human adult normal brain (% of stained cells)/ staining intensity (score 1 to 3) (The Human Protein Atlas*)			
		Neurons	Glia	Neuropil
Olig2	%	0	<25 %	0
	Score		1	
PTPRZ1	%	25–50 %	25 %	>75 %
	Score	2	3	1
ID4	%	>75 %	<25 %	>75 %
	Score	3	2	1
Olig1	%	>75 %	>75 %	>75 %
	Score	2	2	1
CCND2	%	25–75 %	0	>75 %
	Score	1		1
AQP4	%	<25 %	25–50 %	>75 %
	Score	1	2	3
NCAN	%	0	0	>75 %
	Score			2
Sox2	%	25–75 %	25–75 %	>75 %
	Score	1	2	1

Expression: % of stained brain cells proposed by The Human Protein Atlas—0 %, absent; <25 %, weak; 25–50 %, moderate; >75 %, strong

Score of staining intensity proposed by The Human Protein Atlas—1, low intensity; 2, moderate intensity; 3, strong intensity

[a] These data were extracted in March 2013

Many markers potentially specific for GSCs have been proposed such as A2B5 cell surface ganglioside epitope (*A2B5*) [26, 27], aldehyde dehydrogenase (*ALDH*) [28], BMI1 polycomb ring finger oncogene (*BMI1*) [29, 30], fucosyltransferase 4 (*FUT4*) (CD15) [31], Thy-1 cell surface antigen (*Thy-1*) (CD90) [32], prominin-1 (*PROM1*) (CD133) [5, 6], chemokine (C-X-C motif) receptor 4 (*CXCR4*) [33], inhibitor of DNA binding 1 (*ID1*) [34], integrin α6 (*ITGA6*) [35], L1 cell adhesion molecule (*L1CAM*) [36], maternal embryonic leucine zipper kinase (*MELK*) [37], musashi-1 (*msi1*) [38], nestin (*NES*) [38–40], octamer-binding transcription factor 4 (*OCT4*) [41], *OLIG2* [42] and *SOX2* [38, 43–45]. Among these, much attention has been given to CD133, which is currently used to identify and isolate GSCs [40, 43, 46–50]. Despite the fact that this is the most widely used antigen for enrichment of GSCs, there are several arguments to suggest the existence of CD133-negative GSCs: CD133 is not detectable in many fresh GBM specimens [14, 31, 51], and some studies revealed CD133-negative GBM cultures with the ability to self-renew and to form tumours in xenotransplantation assays [14, 51, 52].

Microarray analysis of gene expression profiling have been source of criticisms [18, 53, 54]; it should be noted, however, that we used data from similar DNA chip microarray platforms allowing comparison of data obtained in the same way. This approach reveals eight genes that are strongly overexpressed in cultures enriched in GSCs compared to serum-cultured GBM cells. Surprisingly, except for *OLIG2* and *SOX2*, these genes do not correspond to know GSC markers; there is, however, a lack of standardisation of

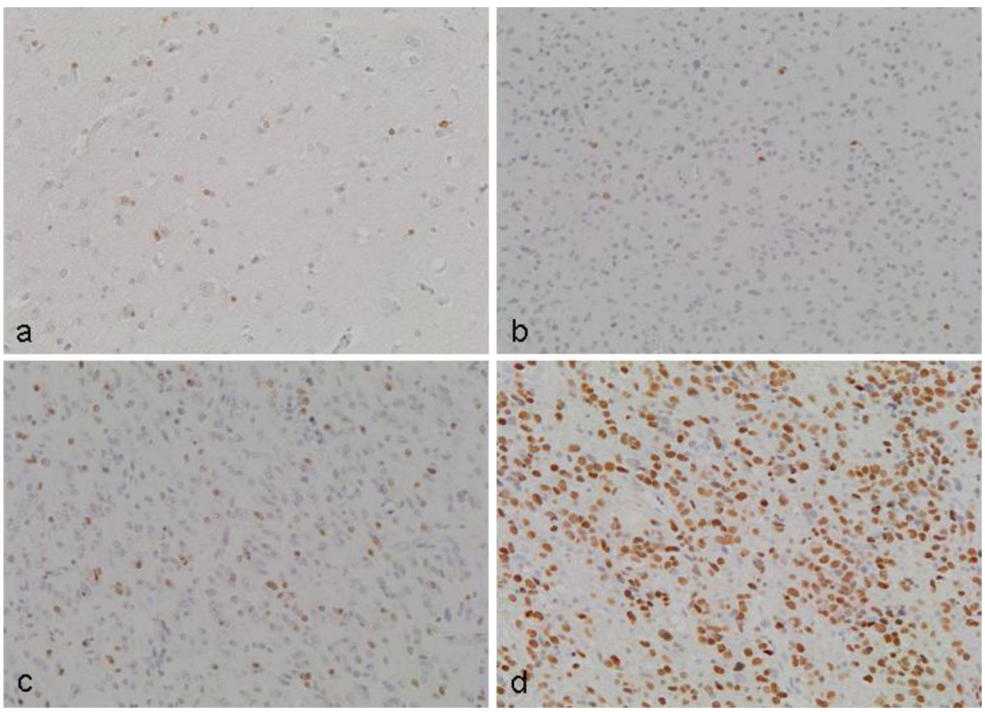

Fig. 1 Olig2 expression profile in glioblastoma and in adjacent peritumoural normal brain (×200). **a** Adjacent peritumoural normal brain. **b** Glioblastoma: <5 % stained tumour cells (+), **c** 5 to 25 % stained tumour cells (++), **d** >25 % stained tumour cells (+++)

Table 5 Distribution of immunohistochemical scores of Olig2 expression according to clinical features

Clinical features	*OLIG2*
Primary glioblastomas (n=9)	+, 5 ++, 2 +++, 2
Secondary glioblastomas* (n=9)	+, 2 ++, 2 +++, 5
Age (years)	
<50 years old (n=9)	+, 3 ++, 3 +++, 3
≥50 years old (n=9)	+, 4 ++, 1 +++, 4
MGMT promoter methylation	
Present (n=13)	+, 4 ++, 3 +++, 6
Absent (n=5)	+, 3 ++, 1 +++, 1
EFGR amplification	
Present (n=10)	+, 4 ++, 3 +++, 3
Absent (n=6)	+, 3 ++, 1 +++, 2
Recurrence within 18 months (n=4)	+, 1 ++, 1 +++, 2

[a] Classification in secondary glioblastomas based on the presence of preexisting histological lesions and/or presence of IDH1 mutation (+, <5 %; ++, 5–25 %; +++, >25 %)

methods for the isolation of GSCs. In particular, cell sorting methods lack standardisation. Moreover, the use of flow cytometry is not ideal because by lack of gold standard, investigators use different markers [55–57].

Moreover, there is still a debate regarding the best method for culturing GSCs. Although in vivo serial transplantation assay is considered the gold standard for identifying cancer stem cells, it remains an arduous and time-consuming method and we need reliable surrogate assays. The neurosphere assay has been used by many laboratories to isolate GSCs. Neurosphere cultures are heterogeneous populations of cells; nevertheless, they maintain the phenotype and genotype of the original tumour better than the same cells cultured as adherent cells under serum-containing conditions [15]. Furthermore, the heterogeneity of neurospheres might mirror the in vivo reality [12].

Thus, the neurosphere assay remains an informative surrogate especially to show the ability of GSCs to self-renew. In this purpose, it is necessary to ensure that GSCs have been isolated by at least demonstrating self-renewal over an extended period of time [12, 58]. The three studies selected in this paper have used the neurosphere assay as method of GSC isolation; all GSC cultures studied in these three studies were at passage >6 (for a total of 20 cultures), except for eight cultures in the study of Lee et al. [15] which were at passage 3 or 5. So, we can assume that the cultures studied were well enriched in GSCs.

Among the eight selected gene candidates, only three gene candidates are found across the three studies (*OLIG2*, *PTPRZ1* and *ID4*). The search for the expression levels of these markers in normal brain has highlighted that *OLIG2* seems the marker with the lowest expression in adult normal brain, and the most specific of glial lineage. The data of the literature confirm that the expression of Olig2 in adult normal brain is restricted to progenitor cells and mature oligodendrocytes [42, 59].

Olig2 is a bHLH transcriptional repressor protein that plays essential roles in the lineage specification of progenitor cells into neuronal subtypes (somatic motor neurons and forebrain cholinergic neurons) and oligodendrocytes during central nervous system development [60–66]. At early embryonic stages, one key role of Olig2 is to maintain progenitor cells in a replication-competent state [67]. The structurally related Olig1 transcription factor is required for maturation of oligodendrocyte progenitors [68, 69]. Although it is co-expressed with Olig2 at early stages, Olig1 function is dispensable for specification of neurons or oligodendrocytes from replication competent progenitor cells [62, 66].

Several lines of evidence suggest that the activity of Olig2 might provide a mechanistic link between growth of malignant glioma and adult neural stem cells. First, a subpopulation of type B and type C cells in the adult rodent brain express Olig2 [61, 70, 71]. Second, exposure to glioma-relevant mitogens, such as EGF or PDGF, stimulates proliferation of Olig2+ rapidly dividing “type C” transit amplifying cells and glioma-like growths [72]. Moreover, all gliomas, irrespective of grade, express Olig2 in at least some fraction of the malignant cell population [42, 73–78]. Thus, our results seem consistent with the literature, in particular with the study of Ligon et al. which showed that Olig2 is a marker of GSCs because Olig2 function is required for glioma formation in a genetically relevant murine model [42].

Furthermore, it is interesting to note that in light of recent molecular and proteomic classifications of GBM [79–81], Olig2 is recognised as a marker of the proneural GBM subtype, which is one of the clinically relevant subtypes of GBM. In parallel, some studies suggested that there are also different subtypes of GSCs and identified two different subtypes of GSC: proneural type and mesenchymal type [82–84]. In the studies of Mao et al. [82] and Bhat et al. [83], Olig2 is recognised as a marker of proneural GSC.

Table 6 Literature review of immunohistochemical expression data in glioblastomas for our eight gene candidates that emerge from our analysis of concordance

Gene	Percentage of stained tumour cells	Number of glioblastomas tested	References
OLIG2	None	4	[77]
	<5 %	9	[78]
	<25 %	13	[59]
	50–70 %	21	[75]
	11–25 %	8	[86]
	50 %	8	[87]
	5–50 %	79	[88]
	0–>50 %	72	[81]
PTPRZ1	65 %	24	[89]
ID4	10–100 %	13	[90]
	>50 %	53	[91]
OLIG1	25 %	11	[73]
CCND2			–
AQP4	20–50 %	79	[92]
NCAN			–
SOX2	>20 %	67	[44]
	12–40 %	12	[93]
	50–100 %	75	[94]

Database of the "National Library of Medicine's PubMed". We used different combinations of the following keywords: "glioblastoma", "gliomas", "immunohistochemistry" and "expression"

Our results are consistent with these data. Indeed, of the three articles used in our study, Schulte et al. [16] identified a distinct subset of glioblastoma stem-like cell lines called GSf lines which displayed a full stem-like phenotype and displayed a proneural gene expression signature. In their data (illustrated in Supplementary Table 1), Olig2 is overexpressed in these GSf lines with an expression ratio of 8.75 compared to 3.66 for the GSr lines, which displayed a mesenchymal gene expression signature.

The second purpose of this work was to study the expression of our gene candidate by immunohistochemistry, an easy method used in pathology daily practice. Although small, our clinical series suggests that the number of Olig2-positive tumour cells is greater in secondary GBM as compared to primary GBM, and two of the four recurrent GBM were strongly positive for Olig2. This may reflect enrichment in GSCs following prior administration of antitumoural treatment. Interestingly, the expression of Olig2 seems to involve a greater percentage of cells in GBM with *MGMT* promoter methylation; *MGMT* promoter methylation status has been introduced as being a predictive biomarker that can be used for stratification of treatment regimes [85].

An important part (7/18 cases) of our tumour samples present more than 25 % stained tumour cells, consistent with data from the literature as described in Table 6. Cancer stem cells are supposed to be rare within a tumour; in literature, the percentage of GSCs is considered to vary between 2 and 5 % of tumour cells although some studies report higher percentages, up to 60 % [6, 13, 95]. This discrepancy could be explained by several ways including the complex morphological and biological heterogeneity of GBM, the lack of standardisation of methods used for the GSC isolation like mentioned above and the fact that the series used to study markers are often small.

Nevertheless, if markers identified in our analysis are indeed consistent markers of GSCs, the opinion that GSCs represent only a small contingent of tumour cells might be erroneous.

In conclusion, our study, starting from comparative analysis of DNA chip microarray data, suggests *OLIG2* is the most specific GSC marker. This needs, however, confirmation by additional investigations with careful considerations about methodology and strategies of validation in particular by serial in vivo transplantation assays. If confirmed, Olig2 forms a tool for detection of GSCs and for development of targeted therapies.

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Conflicts of interest None

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