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Glioblastoma Stem Cells: Conversion or Reprogramming from Tumor Non-Stem Cells?

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Abstract

It is widely accepted that gliomas origin from immature glia and the most important hypothesis is that this origin is from glioblastoma stem cells (GSCs). GSCs are responsible for tumor growth, proliferation, therapy resistance and recurrence. They may represent transformed normal neural stem cells (NSCs), embryonically regressed adult glia or, simply, a functional status that could be regulated by the tumor microenvironment. Objective of the work is to interpret all the immunohistochemical, genetic and *in vitro* culture features of primary tumors and cell lines in favor of the above mentioned hypothesis on the functional status and microenvironment. A series of glioblastomas (GBMs) have been studied after stereotactic biopsies for expression of stemness and differentiation antigens, genetic aberrations and stem cell generation potential by immunohistochemical, immunofluorescence, molecular genetics methods. Perivascular and perinecrotic niches are the crucial points where microenvironment exerts its influence. The most malignant regions of GBM contain hyperproliferating areas expressing stemness antigens, such as Nestin, SOX2, CD133 and almost no differentiation antigens and show a high proliferation index. Circumscribed necroses develop within these areas by ischemia due to the imbalance between the high proliferation rate of tumor cells and the low one of endothelial cells. Perinecrotic GSCs are interpreted as elicited by hypoxia through HIF-1/2 constituting thus a niche. The hypothesis can be formulated that the stem cell status is a functional one that can be reached by dedifferentiated tumor cells through embryonic regression and that GSCs surrounding circumscribed necroses may of course represent a niche, but they are the residue of GCSs/progenitors originally populating the hyperproliferating areas. A conversion of tumor non-stem cells into tumor stem cells is possible, as well as reprogramming of tumor cells due to the microenvironment regulation through its intrinsic and extrinsic signaling. This hypothesis could influence the therapeutic strategies addressed to annihilate GSCs.

Keywords: Glioblastoma; Microenvironment; Stem cells; Niches dedifferentiation; Conversion; Reprogramming

Abbreviations: AC: Adherent Cell; bFGF: basic Fibroblast Growth Factor; CE: Contrast Enhancing; CSC: Cancer Stem Cell; DAPI: 4',6-Diamidine-2'-Phenylindole, Dihydrochloride; EGF: Epidermal Growth Factor; EGFR: Epidermal Growth Factor Receptor; FBS: Fetal Bovine Serum; FFPE: Formalin Fixed, Paraffin Embedded; FOXM1: Forkhead Box M1; GBM: Glioblastoma; GalC: Galactocerebroside; gDNA: Genomic DNA; GFAP: Glial Fibrillary Acidic Protein; GSCs: Glioblastoma Stem Cells; H&E: Haematoxylin and Eosin; HGF: Hepatocyte Growth Factor; HIER: Heat-Induced Epitope Retrieval; HIF-1α: Hypoxia-Inducible Factor-1α; HPF: High Power F ield; IF: Immunofluorescence; IHC: Immunohistochemistry; LI: Labeling Index; MGMT: O^6-Methylguanine-DNA Methyltransferase; MI: Mitotic Index; MRI: Magnetic Resonance Imaging; NE: Non-Contrast Enhancing; NPCs: Neural Progenitor Cells; NS: Neurosphere; NSA: Neurosphere Assay; NSCs: Neural Stem Cells; Oct4: Octamer-Binding Transcription Factor 4; OPCs: Oligodendroglial Precursor Cells; PcG: Polycomb Group; PDGFRA: Platelet-Derived Growth Factor Receptor, alpha; POU3F2: POU Class 3 Homeobox 2; PTEN: Phosphatase and Tensin Homolog; REST: Repressor Element-1 Silencing Transcription Factor; ROIs: Regions of Interest; SALL2: Spalt-Like Transcription Factor 2; SOX2: Sex Determining Region Y-box 2; STR: Short Tandem Repeat; Tcf-4: Transcription factor-4; TERT: Telomerase Reverse Transcriptase; TMZ: Temozolomide; TP53: Tumor Protein p53

Introduction

The existence of a similarity between cancer cells and embryonic stem cells is known since Virchow. Glioma cells may derive by transformation from immature glia cells [1,2], primitive neuroepithelial cells or neural stem cells (NSCs) [3,4] that give origin to glioblastoma stem cells (GSCs) sharing proliferation capacity and self-renewal with NSCs [5] and genetic/epigenetic alterations with malignant gliomas [6]. The transformation of NSCs or neural progenitor cells (NPCs) into GSCs [7] takes place either during embryogenesis or during migration. Generally, with the term GSCs a heterogeneous pool of stem cells and progenitors is meant, even originating from cell dedifferentiation. A recent new possibility is the origin from oligodendroglial precursor cells (OPCs) or NG2 cells [8-11].

GSCs are conceived in the tumor as a special cell population responsible for tumor growth, recurrence, resistance to radio- and chemotherapy and for the failure of the local tumor control. From a therapeutic point of view, the main problem, therefore, concerns their location in the tumor and how to reach them. In malignancies, it has been demonstrated that cancer stem cell (CSC) enrichment may occur in the tumor either from an increased symmetric self-renewal division rate or from a reprogramming of non-CSCs to CSCs that

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confers plasticity to the tumor cell composition [12]. It can occur also after classic anticancer treatments including radiotherapy [13] that increases the number of CSCs through advanced DNA damage repair mechanisms, survival and subsequent expansion of the (more resistant) quiescent fraction of CSCs as they return to a proliferative state [14]; a switch from asymmetric to symmetric CSC self-renewal division [13,15,16] and faster cell cycling of CSCs may occur as well [13]. Also treatment with temozolomide (TMZ) can shift non-GSCs towards GSCs that become positive for CD133, sex determining region Y-box 2 (SOX2), octamer-binding transcription factor 4 (Oct4) and Nestin in a kind of inter-conversion due to chemotherapy [17].

The inter-conversion between CSCs and non-CSCs could be due to environmental factors to keep active their dynamic equilibrium by bidirectional regeneration mechanisms of CSCs through epigenetic mechanisms [18]. In gliomas such equilibrium would involve the relationship among GSCs, NSCs, OPCs, the anatomical site and the microenvironment [19]. The GSC population is a heterogeneous mixture of stem cells and more or less differentiated progenitors. Distinct subsets are inter-related, hierarchically organized as the basis for plasticity [20].

Mature glia cells can dedifferentiate under special conditions and acquire stemness properties [21,22]; in the same way, glioblastoma (GBM)-differentiated cells may be able to dedifferentiate towards a stem-like status when submitted to appropriate stimuli [23] that can be hypoxic conditions, hepatocyte growth factor (HGF), TMZ [17,24,25] and irradiation. In human primary GBM cell lines, a sub-toxic irradiation dose can induce at long term the over-expression of a large panel of stem markers in GBM cells, a potentiation of their neurosphere (NS)-forming capacity and an exacerbated tumorigenesis in nude mice [26]. Irradiation in GBM may cause a phenotypic shift towards stemness, an increase of heterogeneity and aggressiveness, and recurrence [27].

The GSC origin is a widely shared hypothesis [28]. We have previously hypothesized that they might originate from dedifferentiation of tumor cells that takes place in the most malignant tumor areas under the influence of tumor microenvironment [29] with its crucial perivascular and perinecrotic niches. The aim of the present work is to interpret in terms of re-conversion the dynamics that links the occurrence of GSCs in perinecrotic niches and in highly proliferating areas, based on pathological, molecular, immunohistochemical and *in vitro* culture observations in human GBM samples.

Material and Methods

Histology

Thirty GBMs were studied, operated on at the Neurosurgery Unit of CTO Hospital/Città della Salute e della Scienza (Turin, Italy). In each case, before operation, tumor samples were stereotactically removed: one from contrast enhancing (CE) area and two from non-contrast enhancing (NE) areas, as indicated by ROIs (regions of interest) on magnetic resonance imaging (MRI) applied to the StealthStation® Treon Plus™ Surgical Navigation System (Medtronic, Minneapolis, MN, USA), according to a previously described procedure [30]. Surgical tumor specimens were split in three consecutive fragments: the first one was formalin fixed, paraffin embedded (FFPE) as the whole removed tumor, the second one was minced and enzymatically dissociated for expansion in culture, and the third fragment was stabilized in RNAlater® solution (Thermo Fisher Scientific Inc., Waltham, MA, USA) and frozen at -80°C for molecular genetics.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Immunohistochemistry (IHC)

On 5 µm thick paraffin sections, beside haematoxylin and eosin (H&E), IHC were performed on a Ventana Full BenchMark® XT automated immunostainer (Ventana Medical Systems, Inc. Tucson, AZ, USA). Heat-induced epitope retrieval (HIER) was performed in Tris-EDTA, pH 8 and the UltraView™ Universal DAB Detection Kit (Ventana Medical Systems, Inc.) was used as detection system. The primary antibodies were the following: mouse monoclonal Ki-67/MIB-1, mouse monoclonal glial fibrillary acidic protein (GFAP) (from Dako Denmark A/S, Glostrup, Denmark), rabbit polyclonal Nestin (Merck Millipore, Bedford, MA, USA), mouse monoclonal SOX2 (R&D Systems, Minneapolis, MN, USA), rabbit polyclonal repressor element-1 silencing transcription factor (REST) (Bethyl Laboratories, Inc., Montgomery, TX, USA), rabbit polyclonal Survivin, mouse monoclonal hypoxia-inducible factor-1α (HIF-1α), rabbit polyclonal transcription factor-4 (Tcf-4) (from Novus Biologicals, Littleton, CO, USA), mouse monoclonal Oct4 (Abcam, Cambridge, UK), mouse monoclonal CD34 (Ventana Medical Systems, Inc.) and mouse monoclonal Cyclin D1 (NeoMarkers, Fremont, CA, USA). The labeling index (LI) of Ki-67/MIB-1 nuclei was calculated as the percentage of positive nuclei in comparison with the total number of nuclei in 1000x high-power field (HPF) with oil immersion.

In vitro cultures

Fresh surgical tissue was processed as previously described [6]. Culture conditions were: Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (bFGF) for neurosphere assay (NSA) and DMEM supplemented with 10% fetal bovine serum (FBS) for adherent cell (AC) development. Cell cultures were maintained in a 5% O_2 and 5% CO_2 humidified atmosphere. The NS ability to self-renewal, multipotency, clonogenicity and tumorigenicity was assessed as described [6]. Cell line authentication from the respective solid tumor was obtained by Short Tandem Repeat (STR) profiling. Cultures were checked for Mycoplasma contamination before experimental use (e-Myco™ Mycoplasma PCR Detection kit, iNtRON Biotechnology Inc., Gyeonggi-do, South Korea Korea).

Immunofluorescence (IF)

On cryostat sections from frozen sample, IF for CD133/1 (mouse monoclonal AC133 antibody, from *Miltenyi Biotech.,* Bergisch Gladbach, Germany), Musashi-1 (rabbit polyclonal antibody from Merck Millipore), GFAP, Nestin, galactocerebroside (GalC) and βIII-tubulin (mouse monoclonal antibodies from Merck Millipore) was carried out, as previously described [6]. The secondary antibodies used were Alexa Fluor® 488-conjugated AffiniPure goat anti-rabbit IgG and Alexa Fluor® 594-conjugated AffiniPure rabbit anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cell nuclei were stained with 4',6-diamidine-2'-phenylindole, dihydrochloride (DAPI). Negative controls were obtained by omitting the primary antibody. Images were obtained on a Zeiss Axioskop fluorescence microscope (Karl Zeiss, Oberkochen, Germany) equipped with an AxioCam5MR5c and coupled to an imaging system (AxioVision Release 4.5, Zeiss).

Molecular genetics

Molecular genetics was performed on genomic DNA (gDNA) from frozen biopsy specimens and cell lines. gDNA was isolated using the QIAamp DNA Mini kit (Qiagen Inc., Valencia, CA, USA). Allelic imbalances for the critical regions 10q25-26, 9p21 and 17p13, 1p/19q chromosome status, phosphatase and tensin homolog (PTEN), tumor protein p53 (TP53) and telomerase reverse transcriptase (TERT) promoter mutations, epidermal growth factor receptor (EGFR) gene amplification and O^6-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation, were assessed as previously described [31].

Results

Histology

Non-contrast enhancing (NE) areas are characterized by cell differentiation, regressions or cell infiltration. The contrast-enhancing (CE) areas show high cell density, nuclear atypias, high mitotic index (MI) and labeling index (LI) of Ki-67/MIB-1, high vessel density and circumscribed necroses. CE zones can be devoid of small vessels for insufficient angiogenesis (Figure 1A) and circumscribed necroses develop from hypercellular areas (Figure 1B), leaving a perinecrotic palisading as a residue (Figure 1C).

IHC and IF

The hyperproliferative areas show the highest Ki-67/MIB-1 LI of the tumor and they strongly express Nestin, SOX2, REST (Figure 1D-1H) [32], Survivin, hypoxia and HIF-1α, Oct4, Tcf-4 (Figure 2A-2D) with almost no GFAP (Figure 1D). The same features are found in perinecrotic palisadings. In both, CD133 and Musashi-1 expressing cells are demonstrable (Figure 2E and 2F).

Cell lines

NS and AC develop from the samples drawn from CE areas, whereas they develop in a reduced number or with a lower growth rates or do not develop at all from samples removed from more peripheral non-enhancing areas (Table 1).

Molecular genetics

The majority of the genetic/epigenetic alterations are found in biopsy specimens from CE regions, corresponding to the molecular signature of the entire solid tumor. Their spectrum reduces in sample from NE to the tumor periphery, until they can no longer be detected, even in the presence of tumor infiltration (Table 2). NS totally mimic the genotype of the solid tumor that, on the contrary, is absent or only partially present in AC.

Discussion

Circumscribed necroses in GBM have been conceived as developing after ischemia due to small vessel obstructive alterations [33-35]. However, an additional interpretation was that they can develop in tumor areas with a high proliferation rate after ischemia due to an insufficient angiogenesis, because of the imbalance between the high proliferation capacity of tumor cells and the slow one of endothelial cells; pseudopalisadings were interpreted as representing the cells spared by necrosis [36]. The cells of hyperproliferative areas strongly express proliferation and stemness antigens and very poorly differentiation antigens and they contain the highest expression of genetic/epigenetic alterations and stem cell potential. Hypothetically, it might be deduced that malignant transformation implies cell dedifferentiation, embryonic regression and the acquisition of various stemness degrees. The cells with stem cell/progenitor features demonstrable at the borders of the necroses, already described as GSCs evoked by hypoxia through HIF-1α [37-39], might represent the residue of those that previously populated the hyperproliferative areas as dedifferentiated tumor cells become tumor stem cells [29].

As a matter of fact, hyperproliferative areas occur in the phenotypically most malignant part of the tumor, with the highest LI for proliferation markers, i.e. near central necrosis [40]. This is the place where the microenvironment through its internal and external signaling, i.e. genetic and epigenetic mechanisms [23,39,41], exerts its maximum influence that translates into the conversion of highly malignant non-stem tumor cells into tumor stem cells (Figure 3A and 3B).

The CE areas of GBM are also those where the generation of GSCs/progenitors in culture is maximum. Contrary to observations that GSCs can be found everywhere in the tumor [42], they have been described as displaying different characteristics according to the tumor sites [43] or as to be concentrated at the limit between central necrosis and the proliferation zone [6,29,40], where the genetic/epigenetic alterations are fully represented. By microarray, genes associated with mitosis, angiogenesis and apoptosis clustered in this zone [44]. Allelic imbalances, EGFR gene amplification, PTEN, TP53 and TERT

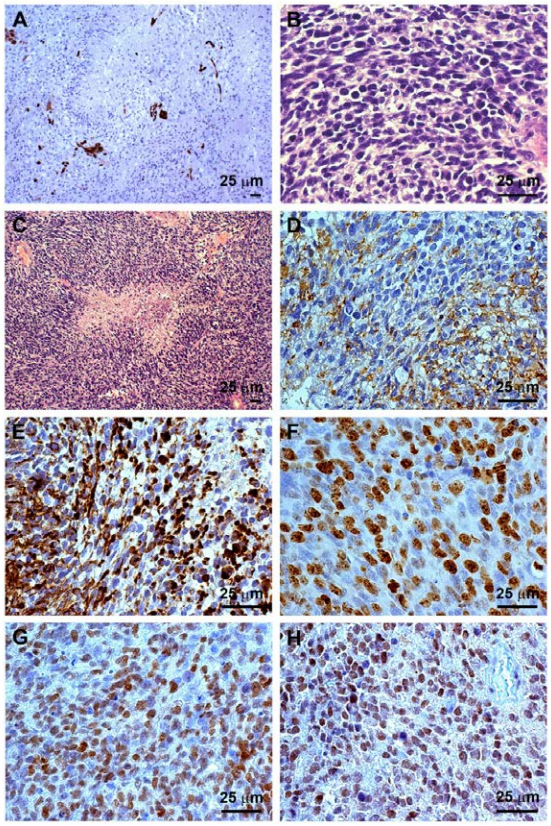

Figure 1: Glioblastoma. (A) Circumscribed necroses develop in a zone poor of small vessels (CD34 immunostaining, DAB), from (B) hypercellular areas (H&E), leaving (C) pseudopalisading as their residue (H&E). (D) Hypercellular areas are poor in GFAP expression (DAB), with (E) high Nestin expression (DAB), (F) high Ki-67/MIB-1 labeling index (DAB), (G) high SOX2 expression (DAB) and (H) high REST expression (DAB). Scale bar: 25 µm.

mutations, and MGMT methylation are more frequent in CE than in NE areas with a good correlation with MRI variables [30]. GSCs/progenitors in the inner portion of GBM, not only express CD133, but they show high level of MGMT [45]. All this is in line with the concept that tumor cells differ each other for phenotypic and molecular characteristics [46].

Our hypothesis is, therefore, that in the hyperproliferative areas of GBM an inter-conversion occurs between non-stem tumor cells and GSCs, induced by the microenvironment. On the other hand, it is already known that cancer progenitor cells can dedifferentiate and acquire a stem-like phenotype in response to either genetic manipulations or environmental cues. They may co-exist with relatively differentiated progenitors in a dynamic equilibrium and may be subjected to bidirectional conversion. The major role would be played by microenvironment and epigenetic patterns [25]. Response to hypoxia means the activation of HIF-1/2 to enhance the self-renewal activity of CD133-positive cells and to inhibit the induction of CSC differentiation [47].

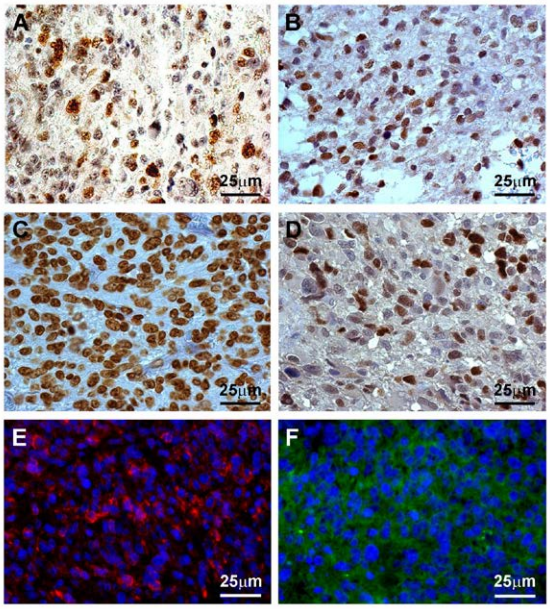

Figure 2: Glioblastoma. (A) Hyperproliferative tumor area expressing Survivin. (B) Id. with high HIF-1α expression. (C) Id. with high Oct4 expression. (D) Id. with high Tcf-4 expression. All DAB. (E) Id. with high CD133/1 expression, immunofluorescence, cryostat sections. (F) Id. with high Musashi-1 expression, immunofluorescence, cryostat sections. Scale bar: 25 µm.

MRI/Histologic pattern	Neurospheres	Adherent cells
CE tumor	++	+
NE tumor	+	+
Tumor with necrosis	–	–
High infiltration	+/–	++
Mild infiltration	+/–	++
Edema with or without infiltration	–	–

MRI: magnetic resonance imaging; CE: contrast enhancing; NE: non-contrast enhancing.
Note: For neurospheres, the scale refers to their number, size and growth rate. For adherent cells, the scale refers as follows: +, temporary monolayer; ++, stabilized monolayer.

Table 1: Development of neurospheres and adherent cells with respect to MRI and the different pathologic features.

Histology	MRI	LOH^{9p,10q,17p}	1p/19q$^{Co\text{-}Del}$	EGFR^{Ampl}	PTEN, TP53, TERT^{Mut}	MGMT^{Meth}
Enhancing tumor	CE	+	+	++	++	+
Tumor infiltration with MVP	CE/NE	+	+	+	+	+
Tumor infiltration	CE/NE	+/–	–	+	–	–
Edema with or without infiltration	NE	–	–	–	–	–

MRI: magnetic resonance imaging; MVP: microvascular proliferation; CE: contrast enhancing; NE: non-contrast enhancing; LOH: loss of heterozygosity: EGFR: epidermal growth factor receptor; PTEN: phosphatase and tensin homologue; TP53: tumor protein p53; TERT, telomerase reverse transcriptase; MGMT: O^6-methylguanine-DNA methylatransferase.
Note: LOH: The scale corresponds to the occurrence (+) or absence (–) of LOH on the tested regions; EGFR: The scale corresponds to the number of samples with gene amplification (++, 100%; +, <50%; –, no amplification); PTEN, TP53, TERT: The scale corresponds to the number of samples with gene mutations (–, wild type); MGMT: The scale corresponds to the number of samples with (+) or without (–) promoter methylation.

Table 2: Comparison among histologic patterns, MRI and molecular genetics.

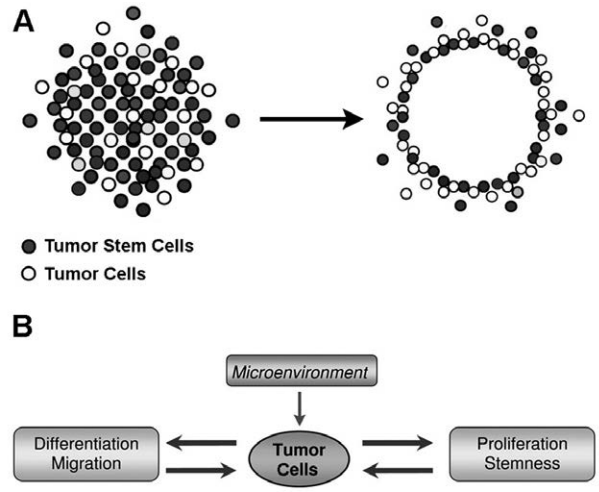

Figure 3: Development of circumscribed necrosis (A). Equilibrium between tumor cells and tumor stem cells (B).

In mammary tumors, oncogenic transformation enhances the spontaneous conversion, so that non-CSCs give rise to CSCs *in vitro* and *in vivo* [48] and CSCs can switch from and to differentiation [49]. In GBM, the factors that could regulate the properties and dedifferentiation of tumor cells to GSCs are not well understood [18,19], even with the recently described perivascular and perinecrotic niches [23,29,40]. For instance, type 1 collagen could be an important niche component for CD133-positive GSCs and to maintain them in adherent culture. Among transcription factors, POU class 3 homeobox 2 (POU3F2), SOX2, Spalt-like transcription factor 2 (SALL2), OLIG2 and Forkhead box M1 (FOXM1) and, among mechanisms of epigenetic regulation, chromatin remodeling, histone modification and DNA methylation, Polycomb group (PcG) proteins have been considered [50].

Perinecrotic and mainly perivascular niches, conceptually sketched [23,39,41] and neuropathologically defined [29], are the starting points of tumor proliferation, angiogenesis and diffusion. GSCs can

be induced or maintained in perivascular niches through endothelial cells and Notch signaling, and in perinecrotic niches through hypoxia and HIF-1/2. Inter-conversion and dedifferentiation could take place always under the regulation of tumor microenvironment that differs from region to region of the tumor [51,52]. Tumor regional heterogeneity concerns hypoxia, genomics, tumor-specific metabolic reprogramming, neo-vascularization or angiogenesis, and stromal immune cells [50]. In this regard, CE areas show different cellular and molecular characteristics compared to tissue from NE margins of the tumors [29,52]. An integrated genomic analysis uncovered extensive intra-tumor heterogeneity, with most patients even displaying different GBM subtypes within the same tumor [53]. Moreover, the reconstruction of the phylogeny of the fragments taken from different tumor regions, identified copy number alterations in EGFR and CDKN2A/B genes as early events, and aberrations in platelet-derived growth factor receptor, alpha polypeptide (PDGFRA) and PTEN genes as later events during cancer progression. The clonal organization of each tumor fragment at the single molecular level detects multiple coexisting cell lineages. This genome-wide architectural intra-tumor variability in GBM across multiple spatial scales and patient-specific patterns of cancer evolution has consequences for treatment design [54].

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