Glioblastoma Unique Features Drive the Ways for Innovative Therapies in the Trunk-branch Era

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Abstract

Glioblastoma multiforme is a solid tumor with particular aspects due to its organ of origin and its development modalities. The brain is very sensitive to oxygen and glucose deprivation and it is the only organ that cannot be either transplanted or entirely removed. Furthermore, many clues and recent indirect experimental evidence indicate that the micro-infiltration of the whole brain parenchyma occurs in very early stages of tumor bulk growth or likely even before. As a consequence, the primary glioblastoma (IDH-wildtype, WHO 2016) is the only tumor where the malignant (i.e. distantly infiltrating the organ of origin) and deadly (i.e. leading cause to patient's death) phases coincide and overlap in one single phase of its natural history. To date, the prognosis of optimally treated glioblastoma patients remains dismal despite recent fundamental progress in neurosurgical techniques which are enabling better maximal safe resection and survival outcome. Intratumor variegated heterogeneity of glioblastoma bulk due to trunk-branch evolution and very early micro-infiltration and settlement of neoplastic cells in the entire brain parenchyma are the reasons for resistance to current therapeutic treatments. With the aim of future innovative and effective therapies, this paper deals with the unique glioblastoma features, the appropriate research methods as well as the strategies to follow to overcome current causes of resistance.

Key words:
glioblastoma unique aspects, trunk-branch era, research methods, causes of resistance, novel therapies

INTRODUCTION

The demonstration that tumor development evolves through a branched rather than a linear pattern is recent and of fundamental importance. The trunk-branch evolutionary framework was first documented by Greaves and colleagues in a paper of 2011 regarding leukemia and subsequently validated also in renal and pancreatic cancers. More recently, it has been confirmed in other tumor types including glioblastoma. The trunk-branch evolutionary growth is due to progressive genomic alterations caused by spontaneous tumoral genetic instability and mitotic errors and driven by selective pressures leading to multiple coexisting neoplastic subclones with different genotypes and phenotypes known as intratumor heterogeneity. Indeed, every tumor mass is not homogeneous but consists of multiple regionally separated subpopulations with variegated genetic, epigenetic and phenotypic features which continuously evolve and adapt during tumor development. Intratumor multifaceted architecture has an immediate consequence in cancer research because it implies that a tumor approached and interrogated by a single biopsy leads to sampling bias, fatally underestimating the complexity of the malignancy. For this reason, the study and analysis of a bulk tumor must invariably begin by collecting multiple multiregional spatially separated samples throughout the whole tumor mass during
surgery or in fresh autopsies. Adding more complexity is the proven evidence that multiple coexisting neoplastic cell populations are present even within a single biopsy. Such a variegated intratumor heterogeneity, due to a multitude of regionally different genetic and epigenetic subclones which evolve over time, is one of the reasons of treatment failure in malignant cancers and it represents a major challenge in preventing the emergence of drug resistance and in developing innovative therapies.

As proposed by several authors, the dynamic growth and progression of a malignant tumor can find an effective analogy with a growing tree. Indeed, recent genetic and molecular studies have demonstrated that tumor development evolves from the founding clone, corresponding to the trunk of the tree, through a branched pattern of growth leading to a spatiotemporal evolutionary intratumor heterogeneity (Fig. 1). However, the latter, currently

Figure 1. Iconic Darwin's drawings (a, from notebook B, 1837-38; b, dated 1868, from material for preparation of the book “The descent of man”, 1871), depicting diversification and selection of species and species-populations during natural evolution, share astonishing similarity with the current and useful analogy that compares the trunk-branch evolutionary tumor development to a growing tree (c). Tumors continuously evolve over space and time from their founding or ancestral clone (the trunk) through a branched pattern of progression leading to a multitude of genetic, epigenetic and molecular variegated subclonal populations (major and minor branches).

The trunk-branch tumor framework

In 1976, the American pathologist and cancer cytogeneticist Peter Nowell published a cornerstone paper where he asserted that cancer growth is subject to an evolutionary process. He was the first who clearly understood and explained that the Darwinian laws of selection applied correctly to tumor development. Cancer cells have a high rate of spontaneous stochastic genetic and epigenetic heritable alterations due to their genetic instability and mitotic errors. As a consequence, an increasing number of cell sublines with different genomic variants are produced during tumor growth and progression leading to a continuously more complex intratumor heterogeneity over time. At the beginning, tumor growth occurs in an ideal microenvironment, well oxygenated and supplied by the original vasculature. It is with natural worsening conditions of the tumor’s microenvironment (onset of hypoxia, decrease in glucose and nutrients, limited space or natural anatomical restraints ...) that among the multiple subclones present in the growing tumor mass, those which possess genetic lesions and or epigenetic modifications conferring advantageous features to resist and survive are selected and will expand. Subsequently, the same laws of selection will apply when the multiple subclones of a neoplasm are challenged by the therapeutic strategies (radiotherapy, chemotherapy, immunotherapy and others) introduced by physicians.

Indeed, by comparing the genomic alterations of the multiple samples and recognizing and distinguishing those which are “ubiquitous” (present in all samples), “shared” (present in several samples but not all) and “private” (present only in one single sample) the spatiotemporal evolution of the tumor can be reconstructed. Thus, the multiple sampling from spatially separated regions of the tumor, at a specific time point, can allow us to map the geography of the bulk and reconstruct its phylogenetic tree going back in time until the founding clone. Whenever multiple samples, not only multiregional in space but also longitudinal in time, are obtained (e.g. longitudinal surgical or biopsies procedures in solid tumors at diagnosis, recurrences and metastatic locations; longitudinal blood samples in hematological cancers; using animal models), the evolution of subclones can be followed and monitored over time. The collection and analysis of multiple spatiotemporal separated samples are therefore crucial in the study of malignancies because a single sample is only a

an insuperable obstacle for therapeutic treatments, has been helpful in inferring and reconstructing the phylogeny of the tumor mass when it was interrogated by the collection and analysis of multiple multiregional separated tumor samples. Indeed, by comparing the genomic alterations of the multiple samples and recognizing and distinguishing those which are “ubiquitous” (present in all samples), “shared” (present in several samples but not all) and “private” (present only in one single sample) the spatiotemporal evolution of the tumor can be reconstructed. Thus, the multiple sampling from spatially separated regions of the tumor, at a specific time point, can allow us to map the geography of the bulk and reconstruct its phylogenetic tree going back in time until the founding clone. Whenever multiple samples, not only multiregional in space but also longitudinal in time, are obtained (e.g. longitudinal surgical or biopsies procedures in solid tumors at diagnosis, recurrences and metastatic locations; longitudinal blood samples in hematological cancers; using animal models), the evolution of subclones can be followed and monitored over time. The collection and analysis of multiple spatiotemporal separated samples are therefore crucial in the study of malignancies because a single sample is only a
snapshot in the four-dimensional spatiotemporal progression of the tumor.

From an evolutionary perspective, genomic lesions are classified as “driver” (advantageous), “passenger” (neutral), “deleterious” (disadvantageous) and “mutator” (alterations favoring other genetic lesions). In the reconstructed phylogenetic tree, the trunk represents the founding clone harboring the ubiquitous genetic driver mutations that can be found in every cell of both the primary tumor and metastatic locations. Additional driver events will happen at the origin of the tumor branches, shared by the branch-descendant cell subpopulations and present only in geographically restricted regions of the tumor. Major (or proximal) tumor branches represent early subclones which continuously evolve over time splitting into minor (or distal) branches and originating a multitude of minority subclones. At all stages during tumor development a high number of passenger and some deleterious and mutator events happen giving rise to an increasingly complex intratumor heterogeneity (Fig. 1).

**Glioblastoma cancer stem cells (CSCs) in the trunk-branch tumor evolution**

The CSC hypothesis, experimentally validated in the last two decades in relation to glioblastoma and many other cancer types, implies that self-renewing CSCs initiate and maintain tumor development. CSCs have the ability to proliferate long-term, self-renew and differentiate as well as remaining quiescent in G0 state, forming tumorspheres in culture due to their clonogenic capacity and giving rise to a tumor phenotypically similar to the parental one when transplanted in recipient mice. CSCs, due to their self-renewal capacity, long-term replicative potential and extensive heritability have been recognized and named as the actual “units of selection” during tumor progression.

Recently, an inspired study has proved that different genetic and epigenetic variants of CSCs exist simultaneously in glioblastomas and that they are longitudinally subjected to the principles of cancer evolution. Given this experimental evidence and their exclusive properties, it is highly conceivable that CSCs drive glioblastoma progression and evolution as cellular drivers of the subclonal expansion. Indeed, CSCs are not fixed entities during tumor development and their adaptability to different oxygen tensions and glucose availability has been unambiguously documented. It derives that natural microenvironmental pressures coupled with spontaneous genomic variations in cells with long-term proliferative potential lead to a dynamic evolution from the founder CSC, which gives rise to the trunk, to other CSCs with genetic and epigenetic modifications that are at the origin and drive the branches progression. Oxygen and glucose availability are undoubtedly the most important physiological forces which continuously select the most suitable subclonal populations in different sectors of the developing bulk tumor. During glioblastoma progression, the ability of tumor cells to adapt to different oxygen tensions has been proven driven by CSCs which subsequently transmit (through asymmetrical division) the advantageous traits to the daughter committed cells. Like other solid tumors, at the beginning glioblastoma growth occurs in ideal microenvironmental conditions. As the tumor expands over time, the original network of cerebral vessels becomes insufficient to supply the growing bulk and in some regions hypoxia begins to develop. The brain, due to its complex functions, is the only organ where normal oxygen tension ranges between 10% and 5%. Thus, while in other parenchymatous organs hypoxia begins at about 1% O2 concentration, in the brain its onset happens initially at about 5% O2. The advent of hypoxia is dramatic for tumor cells and they have to quickly adapt to the worsened microenvironment to survive and continue their progression. It has been demonstrated that in the glioblastoma bulk at about 5% O2 concentration HIF2α is induced and its expression is exclusive of CSCs and not shared by progenitors or committed cells. The latter benefit the adaptive traits (induced in CSCs by HIF2α which gives rise to a complex epigenetic shift) to resist hypoxia and survive as descendants along the differentiating cascade from the CSC subpopulations (also epigenetic traits are inheritable). Being expressed only in CSCs, HIF2α is a CSC marker during the second phase of glioblastoma development ranging from 5% to 1% O2 concentration (phase of mild and severe hypoxia). The first phase is the pre-hypoxic one which ranges from 10% to 5% O2. The third phase is that of very severe hypoxia and ranges from 1% O2 (when also HIF1α is induced) to a minimal level of O2 tension. This clearly indicates that CSCs are the most sensitive cells to the advent of hypoxia (possessing exclusive mechanisms to front it) and transmit the advantageous fitness traits to their progeny driving tumor evolution. Similarly, CSCs have the capacity to quickly up-regulate the Glut3 transporter for glucose uptake in situations of glucose deprivation. Given the described scenario, it is highly conceivable that in the reconstructed tumor phylogenetic tree the founder CSC has given rise to the founding clone corresponding to the trunk. Subsequently, CSC variants (driven by selective pressures or spontaneously developed) of the founder CSC are at the beginning of major and minor tumor branches originating continuously more variegated tumor cell subpopulations over time.

As evolutionary units of bulk tumor development, CSCs are supposed to be the cells which are selected and drive the fatal features of other intransigent malignant cancers as well. Indeed, in relation to other solid tumor types, particular CSC variants are selected through a bottleneck process first acquiring the ability to distantly invade the organ of origin and subsequently colonizing and metastasizing to other organs remaining dormant in the metastatic niches until their activation. While in other solid cancers
to characterize glioblastoma CSCs. In particular, initially, the great controversy regarding CSCs putative markers. Indeed, in the past decade the development of new tumors recapitulating the parental one when transplanted in recipient mice, are well recognized methods to isolate and identify CSCs. On the contrary, controversy exists regarding the capacity to give rise to differentiated progeny and form new tumorspheres and be absent in colonies of cells without the capacity to form spheres. Therefore, the subsequent demonstration that in the adult brain all the founder glioblastoma cells of origin are CD133, that also CD133− cells had bona fide all the features of CSCs and that CD133 was expressed also in progenitors and committed cells shed many doubts about its use as CSC marker in glioblastoma. By definition, a stem cell marker is a marker present in stem cells (undifferentiated cells, sometimes present in early progenitors as well) and lost in progenitors and committed cells (e.g. lost during the differentiation cascade). Furthermore, such a marker must always be present in colonies of cells giving rise to normal spheres or tumorspheres and be absent in colonies of cells without the capacity to form spheres. Therefore, the subsequent demonstration that in the adult brain all the founder glioblastoma cells of origin are CD133−, that also CD133− cells had bona fide all the features of CSCs and that CD133 was expressed also in progenitors and committed cells shed many doubts about its use as CSC marker in glioblastoma. Therefore to date, due to the lack of a recognized exclusive cell surface marker, we still use the previously mentioned functional characteristics to identify the glioblastoma CSCs. Similarly, known stem-progenitor markers related to essential functions of normal stem-progenitor cells (Nestin, Sox2, Bmi1, Musashi1, NANOAG, ALDH1...) are used to identify CSCs in immunohistochemical procedures or stainings on sections.

**Primary glioblastoma: a particular solid tumor**

Until about a decade ago, the prognosis of optimally treated glioblastoma patients was poor with 14.6 months median overall survival. Nowadays, in many centers, glioblastoma surgery is guided by 5-aminolevulinic acid fluorescence (5-ALA) or intraoperative MRI and assisted by neuronavigation technology and neurophysiological monitoring. However, in spite of these recent technical advances, which enable an improved safe resection rate and survival outcome, the prognosis remains still extremely dismal.

Primary glioblastoma, IDH-wildtype within the 2016 WHO classification, accounts for about 95% of all glioblastomas and is a tumor with unique characteristics in relation to other solid malignancies. The particular aspects of the glioblastoma can be traced back to the organ in which it originates and to its development modalities. As mentioned, the brain is the most sensitive organ to lack of oxygen and glucose and hypoxia begins initially at 5% O₂ concentration. The brain is without lymphatic drainage and glioblastomas exceptionally give rise to hematogenous metastases. Indeed, the metastatic spread and the secondary locations of the glioblastoma are inside the brain itself due to migration of neoplastic cells along basement membranes (perivascular, subependymal) and white matter tracts. The brain is the only organ that cannot be either entirely surgically removed or transplanted. As a consequence, the micro-infiltration of the brain parenchyma (and therefore of the organ in which the glioblastoma originates) represents the worst grade of malignancy and definitely the cause of patient’s death.

At this point, a fundamental question arises: Is the “healthy” brain parenchyma micro-infiltrated immediately (in parallel to or even before the growth of the primary bulk) by the CSC of the founding clone, or only subsequently by one (or more) CSC variant (and its downstream cells) giving rise to a particular branch? The correct and conclusive answer will be given by future direct experimental data. However, we have nowadays several important clues and indirect experimental evidence that can address us to correctly anticipate the response.

a) By definition: The primary glioblastoma, as opposed to secondary glioblastoma (IDH-mutant within the 2016 WHO classification), is a malignant tumor, i.e. distantly infiltrative, since its origin. Therefore, it is conceivable that the micro-infiltration of the entire brain parenchyma travels at least in parallel to the growth of the primary bulk tumor from the beginning. However, it is equally plausible and realistic that the spread throughout the whole brain parenchyma and the settlement of CSCs in the perivascular niches even precede the growth of the primary tumor mass. If this concept is exact, we can deduce that the CSC giving rise to the trunk (founder CSC) possessed all the features and capacities to invade the brain parenchyma and subsequently exit the cell cycle and settled quiescent in G₀ state in the perivascular parenchymal niches until activation.

b) Radiologically: Examining T2-weighted and T2-FLAIR MRI sequences, we can get evidence of extensive hyperintensity, reflecting vasogenic edema and suggesting tumor cells infiltration, beyond the T1Gd MRI contrast-enhancing primary lesion, both in the ipsilateral and contralateral hemisphere, even when the primary bulk has been detected of a small size. However, such suggested early invasion around the tumor is supposed to be much larger because the micro-infiltration of single cells or small cluster of cells (in particular founder CSCs settled quies-
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cent in the whole brain parenchyma) cannot currently be
detected by radiological devices. In addition, patterns of
both glioblastoma multifocality at diagnosis and of distant
recurrences for the majority of glioblastomas grouped ac-
cording to the data of the Lim-Berger classification indicate
diffuse micro-infiltration in very early stages of glioblasto-
ma development.44

c) Clinically and surgically: It is well recognized that
even small glioblastomas entirely removed surgically (both
from a contrast-enhancement and fluorescent point of
view) inevitably give rise to either local or distant recur-
rences after a certain period of time. Furthermore, in
the past great pioneers of neurosurgery were allowed and tried
and perimarginal invasive growth. Different is the case of
other solid tumors which become malignant when they
茵and become deadly when they are able to successfully
acquire the capacity to distantly infiltrate the organ of ori-

in primary glioblastoma the malignant and deadly phases
coincide and overlap as a unique phase of its natural his-
story (see below).59,60

Given what mentioned above, awaiting direct experi-
mental evidence, all data unequivocally indicate that the
whole brain parenchyma is diffusely micro-infiltrated from
the beginning of primary glioblastoma development. This
very early brain parenchyma micro-infiltration coupled with
the impossibility to entirely remove the brain are the

Due to the unique features of the brain, the study of the
micro-infiltrated brain parenchyma is undoubtedly the
most difficult task. It can be performed in vitro, from surgi-
cal patient tissue, after correct sampling procedures with
full respect for ethical issues. Alternatively, the analysis of
the micro-infiltrated brain parenchyma can be performed
in preclinical animal models. Amongst these ones, “The de-
velopment tumor model” (TDTM) can enable us to follow
and monitor glioblastoma progression in the whole brain
from the beginning to the end of its natural history (see be-
low).34,35 Fresh autopsies must also be considered, however
the particular rapid decay of the brain parenchyma allows
only DNA analysis procedures, while RNA expression and
proteomic studies are not feasible. Recent papers have ad-
dressed glioblastoma relapses and compared their genetic
alterations to those of the primary bulk.59,60 Interestingly,
they have provided evidence of two distinct patterns of
recurrence and indirect experimental proof of very early
brain parenchyma micro-infiltration (see below).

Research methods to study glioblastoma

Among the many researchers who contributed to the un-
derstanding of glioma tumors, it is right to mention the
German pathologists Rudolf Virchow and Hans-Joachim
Scherer.

Rudolf Virchow (1821-1902) provided the first histo-

morphological description of the multiple features which
characterize glial tumors. Importantly, he was the first to
distinguish two groups of glial tumors (corresponding to
low- and high-grade gliomas) based on their cellularity
and pattern of infiltration (circumscribed or diffusely in-
vasive).61,62

Hans-Joachim Scherer (1906-1945) was the first to rec-
ognize two types of glioblastoma. Indeed, he named prima-
ry glioblastomas those tumors originating “de novo” and
secondary glioblastomas those that developed from a low-
grade astrocytoma. He clearly explained that the two types
of glioblastoma share the same histopathological charac-
teristics but differ largely in their biological and clinical prop-
eties. Furthermore, Scherer was the first to study several
samples of the same primary bulk separately, providing
evidence that the particular glioblastoma features such as
microvascular hyperplasia and pseudopalisading necrosis
are heterogeneously distributed within the tumor mass.63,64

Currently, glioblastoma research studies must abso-
lutely take into consideration both the tumor bulk and the
micro-infiltrated brain parenchyma and can be carried out
analyzing neoplastic and “healthy” material obtained from
living patients (a), using animal models (b), studying post-
mortem samples from rapid autopsies (c), comparing local
and distant recurrences with the primary tumor (d).

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recurrence and indirect experimental proof of very early
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A. Living patients

The research procedures invariably begin in the operating theatre by collecting multiple samples using fluorescence-guided removal of the bulk. Extent of tumor resection and functional preservation are the most important factors influencing the glioblastoma patients’ outcome. In the operating theatre, neurosurgeons always try to achieve maximal tumor removal whilst preserving the “healthy” parenchymal tissue and eloquent brain regions.

Nowa-resection will take into consideration 5-ALA guided surgery. Technical progress, such as spectroscopic devices and near-infrared confocal endomicroscopy, is ongoing to improve the identification of small clusters and isolated tumor cells.

\textit{a1)} the bulk

To sum up, we can trace these steps:

\begin{itemize}
  \item Collection of multiple spatially separated samples (open surgery guided by 5-ALA and assisted by neuronavigation system and neurophysiological monitoring) of the glioblastoma bulk. The way a glioblastoma is removed depends on the situation in the operating theatre. The samples can be collected either in a piecemeal fashion during tumor debulking or from the entire bulk after en bloc removal. Several samples must be obtained, paying attention to include the regions at the core of the tumor and those close to or in contact with the ventricular system (due to the presence of the SVZ and where the most ancestral subpopulations are likely located). The samples must be numbered and their location in relation to the geography (pre-operative MRI and intraoperative fluorescent tissue) of the bulk mapped.
  \item All the samples must be subjected to genetic, epigenetic and molecular analysis (including all “omic” studies, e.g. genomic, transcriptomic and proteomic). Particular attention must be paid to the sample containing the founding clone (if found, after that the phylogenetic tree has been
\end{itemize}
reconstructed) or to that containing the most ancestral one. The epigenetic analysis will enable us to determine the subtype(s) of the glioblastoma. In relation to this, The Cancer Genome Atlas (TCGA) Research Network has revealed four glioblastoma subtypes ( proneural, neural, classical and mesenchymal) and a recent paper has highlighted that some of these subtypes can be present and regionally separated in the same glioblastoma bulk.

- Reconstruction of the phylogenetic tree. The collection and analysis of multiple spatially separated samples can allow us, via the recognition and comparison of ubiquitous, shared and private mutations, to infer and reconstruct the phylogenetic tree of the tumor. At the same time driver and passenger events must be identified. Driver events must be inferred at both the beginning of the tumor (the trunk, corresponding to the founding clone) and of the division branches (subclones).

- The proper methods to culture, identify, isolate and analyze the CSCs of the samples must be used (neurobasal serum-free medium, tumorsphere formation assay, limiting dilution assay, replating one cell per well...). The CSCs deriving from the different samples must be compared to each other in order to get evidence of their genetic and epigenetic heterogeneity inside the bulk at the time of surgery. The driver events of the founder CSC must be clearly inferred and identified. Driver mutations of the founder CSC will be ubiquitous alterations and would be preferred targets for therapy. Unfortunately however, as seen also in other solid tumors, due to pre-existing variant mutations or alternative mechanisms and pathways in minority cell subpopulations at treatment introduction, therapies aimed at targeting some driver events have not achieved effective results so far. Thus, likely only driver events which are stable and without alternative mutations and pathways can be exploited for future effective treatments. Furthermore, for therapy to be effective and doses tolerated the drug(s) should be delivered to selectively target and reach the CSCs and not all cancer cells (including early and late progenitors and committed cells) (see below).

a2) the micro-infiltrated brain parenchyma

The in vitro study of the micro-infiltrated brain parenchyma from surgical patient tissue can occur analyzing samples appropriately taken maintaining full respect for ethical issues. The most favorable situation happens in a few glioblastoma patients. Indeed, when a glioblastoma is confined to and widely invading a frontal or temporal pole or lobe (preferably of the non-dominant hemisphere), frontal or temporal polectomy or lobectomy is definitely the ideal way to achieve complete tumor resection. In these cases, we can obtain the entire removal of the glioblastoma and some regions of micro-infiltrated brain parenchyma around the bulk. This enables us to study and analyze the micro-infiltrated brain parenchyma (in search for the parenchymal niches and quiescent CSCs) at a specific point in time and additionally in the “healthy” parenchymal areas significantly far away from the peritumoral margin of the bulk. These rare cases of polectomy or lobectomy (containing “healthy” brain tissue around the glioblastoma) are therefore precious sources for research purposes because in all other occasions, due to ethical reasons, the neurosurgeon must absolutely limit the resection where the pathological and fluorescent tissue ends. However, it may happen during the surgical removal of the glioblastoma that small amounts of the confining “healthy” peritumoral margin just beyond the fluorescent brain/tumor interface can remain included in the resection and this tissue is of inestimable value for research studies of the micro-infiltrated brain parenchyma. Another possibility arises when glioblastomas are deeply located inside the brain parenchyma. Whenever feasible, they are reached through a trans-sulcal route preserving the cortical and subcortical adjacent brain areas that are enlarged by cottonoids. However sometimes, due to anatomical surgical reasons, a trans-sulcal approach cannot be used and the best and shortest way to reach the tumor is via a transcortical route. Therefore, a thin strip of brain parenchyma must be coagulated and the parenchymal edges separated and enlarged by cottonoids to create a corridor to gain access to the most superficial regions of the tumor. In these occasions, when creating the parenchymal corridor, one or two small samples from the edges of the corridor can be collected before their gentle coagulation needed to obtain hemostasis. Finally, when a neuronavigation-assisted biopsy procedure in inoperable patients is adopted, the first (sometimes) and last (more often) samples collected along the planned biotic trajectory (usually in one spatial dimension comprising all the length of the tumor) may contain both tumor tissue and “healthy” peritumoral margin.

The above mentioned proposed methods to sample “healthy” micro-infiltrated brain parenchyma in the operating theatre are totally permitted ethically as part of normal brain surgery to remove glioblastoma tumors (Fig. 3). The same principles apply to either local or distant recurrences.

The collected samples from the micro-infiltrated brain parenchyma must be subjected to histological and immunohistochemical examination. Flow-cytometry analysis can enable us to determine the DNA index of tumor cells. All the methods previously described to identify the CSCs in the micro-infiltrated brain parenchyma (i.e. the samples may be tested in culture, by transplantation, for side population cells and by stainings on sections with stem-progenitor related markers) must be performed as well as all “omic” studies including single cell DNA and RNA sequencing of the identified CSCs.

B. Animal models

The entire reproduction of the glioblastoma natural history can be achieved using the preclinical animal model “The development tumor model” (TDTM) and orthotopic transplantation of human glioblastoma-derived cells cultured in a neurobasal serum-free medium to obtain tumorspheres enriched in CSCs. Ideally, pre-hypoxic cells of
the founding clone should be transplanted. If the founding clone is not found among the multiple samples collected and analyzed, the most ancestral subpopulations, inferred after reconstruction of the phylogenetic tree, must be used. The model posits the transplantation of the same number of cells into several genetically identical immunocompromised rodents (an inbred strain of mice or rats) at the same time (time zero). Thus, the model creates a pool of twin immunodeficient transplant animals examined under the same conditions. By sacrificing one animal a week (or choosing any time interval as needed) and getting multiple biopsies (from the bulk, ipsilateral hemisphere, corpus callosum, contralateral hemisphere) and stainings on sections during the progression of the xenograft, we can biologically follow the entire development of the glioblastoma both in the primary tumor and in the micro-infiltrated brain parenchyma.34,35 This way, TDTM can allow us to biologically and spatiotemporally (i.e. geographically and longitudinally) reproduce and monitor both the entire evolution in the bulk (appreciating the evolving variegated clonal architecture and increasingly more complex intra-tumor heterogeneity) and the detection of all steps (from the time of transplantation to the end of the natural history) of micro-infiltration of the brain parenchyma. The study of bulk heterogeneity evolution implies that the entire tumor mass must be taken from the sacrificed animals in order to get multiple sampling even if the bulk is quite small. Other biopsies and stainings on sections will enable us to continuously monitor the progression of the brain parenchyma micro-infiltration over the development of the glioblastoma xenograft. Indeed, TDTM can enable us to identify the CSCs in the brain parenchyma shedding light on the perivascular parenchymal niches where founder CSCs have exited the cell cycle and settle quiescent in G0 state until activation. Furthermore, TDTM is perfectly tailored to test new therapies. By delivering at one time point over xenograft progression to all rodents the identical dose and modality of therapeutic treatments, the model can allow us to follow the dynamics, response and emergence of subclones during and after innovative therapies (delivered as either chemotherapy, radiotherapy, immunotherapy or others) both in the bulk and in the micro-infiltrated brain parenchyma.

C. Rapid autopsies

Due to very early alteration and decay of the brain tissue, the study of RNA expression as well as proteins regulation is very limited in autopsy procedures. Indeed, the low RNA quality and integrity also in rapid post-mortem brain samples lead to unreliable data in terms of qualitative and quantitative gene expression profiles.77 Similarly, analysis of proteins and neuropeptides has important limitations due to early degradation.78 As known from archeological and forensic studies, DNA is a very resistant molecule and its analysis can be efficiently performed in autopsy brain samples. Furthermore, within 48 hours of post-mortem interval also epigenetic studies of methylation patterns are feasible and reliable.79 Therefore, despite evident weaknesses in RNA profiling and proteins analysis, rapid autopsies can enable us to elucidate genetic and epigenetic methylation aspects related to both local and distant recurrences and brain parenchyma micro-infiltration.

D. Analysis of glioblastoma recurrences

Recent genetic studies have analyzed glioblastoma recurrences and compared them to their primary bulk.59,60 The results have indicated that there are two main genetic patterns. Indeed, one of them overlaps largely with the primary tumor while the other one shares only limited genetic alterations with the bulk. As known, the great majority of relapses (about 80%) occur locally and they are assumed to develop from micro-residues after subtotal resection and therefore from active CSCs left behind even in several cases with presumed total removal of the fluorescent tissue. In these latter occasions, local recurrence may develop from small anatomically hidden fluorescent regions or small necrotic areas (which are not fluorescent) left behind or due to the fact that fluorescence of single cells (or of very small cell clusters) beyond the tumor/parenchyma interface cannot be detected during surgery. However, in local recurrences a high degree of mutations should (and indeed they do)59,60 match with the primary tumor suggesting a direct expansion from the active residues. More precisely, local recurrences share all the mutations happened in a sector of the primary bulk and possess some new mutations due to their own evolution.

Much more interesting are the less frequent cases of distant relapses. Indeed, true distant recurrences develop far away from the resected primary bulk and often are observed after polectomy or lobectomy (usually in the non-dominant hemisphere) when a glioblastoma is confined to a pole or lobe. In these cases, in theory, the cell giving rise to the relapse should be one of the CSCs migrated at the beginning of tumor growth (or likely even before) which have micro-infiltrated the brain parenchyma and, once found the ideal microenvironment, exited the cell cycle and settled quiescent in G0 state in the perivascular parenchymal niches. Equivalently saying, in these occasions the relapse is supposed to occur from a CSC of the founding clone at the beginning of tumor development (likely during the brain parenchyma micro-infiltration phase before the growth of the primary bulk) which has remained quiescent or dormant for a long time in the parenchymal niche before activation. As a consequence, the cells and clones of true distant relapses must possess the ubiquitous mutations of the founding clone and at the same time not possess the mutations happened during the branched evolution of the primary bulk. However, in addition to the initial founding mutations, they exhibit some new different genetic alterations occurred during their own progression. Therefore, the recent findings that some relapses have a limited degree of mutation overlap with the primary tumor sharing with it only ancestral mutations (the ubiquitous ones)59,60 confirm the previous reasoning. Furthermore, they provide indirect
Experimental evidence of very early whole parenchymal micro-infiltration by founder CSCs which subsequently lie quiescent for a long time in the perivascular niches until activation. Distant recurrences are therefore very interesting sources for additional future research studies of the micro-infiltrated brain parenchyma (Fig. 4).

**The perivascular parenchymal niche**

Neurogenic GFAP+ subependymal astrocytes, known as normal adult neural stem cells (aNSCs), reside quiescent in specialized microenvironments (the aNSC-niche) in the subventricular and subgranular brain zones (SVZ and SGZ) in close relationship with the microvasculature, endothelial cells and soluble factors that maintain and regulate their properties.80 As their normal counterpart, recent studies have unambiguously documented that CSCs lie in a specific milieu in the bulk tumor, around blood vessels and hypoxic-necrotic areas.31-33,81 This is not surprising being the CSCs the most sensitive cells to O2 deprivation and those cells which possess exclusive mechanisms to front hypoxia when it begins to develop.

Several studies have shown in animal models that many cells invade the parenchyma in early stages after neoplastic cells are xenografted.50,51 However, as tumor cells proceed during the differentiation cascade they restrict their lifespan and after some replicative cycles they senesce and die.7,24 Indeed, those studies that have analyzed the micro-infiltrated brain parenchyma beyond the bulk tumor margin in human samples reported only rare neoplastic cells.82-85 Moreover, recent work has shown and demonstrated that CSCs coming from the glioblastoma peritumoral regions behave differently from those of the tumor mass and locate scattered around blood vessels once xenotransplanted in preclinical animal models.85 It follows we can deduce and reasonably speculate that after the early invasion and micro-infiltration of the whole brain parenchyma, only the CSCs which exited the cell cycle and settled quiescent in a specific perivascular milieu in the whole brain parenchyma until activation. With regards to the cell of origin, the founder CSC derives likely from an aNSC or from an early progenitor. Theoretically, primary glioblastomas can also derive from committed cells. However, due to the need for multiple sequentially ordered steps to dedifferentiate and the clinical-radiological evidence of many primary multifocal glioblastomas, it is unlikely they originate from differentiated cells. Diversely, differentiated cells are likely at the origin of low-grade gliomas that over time acquire genomic and transcriptomic alterations which lead to secondary glioblastomas. If the founder CSC of primary glioblastomas comes from an aNSC, we can deduce that the oncogenic process alters the adhesion or anchorage to the physiologic niche downregulating the related
adhesion receptor genes and at the same time fostering the expression genes linked to migration and proliferation ability. Once they find an ideal perivascular parenchymal compartment, they are likely to underexpress the genes related to migrating and proliferating activities and overexpress the genes for adhesion and anchorage to the new tumoral niche simultaneously exiting the cell cycle and acquiring the quiescent G₀ state. If the founder CSC derives from an early progenitor, which is an active dividing and migrating cell and still possesses self-renewal capacity, the above mentioned first step is not necessary.

Causes of resistance to current therapeutic treatments in glioblastoma

Glioblastoma is characterized by phenotypic diversity, intratumor heterogeneity and treatment failure with dismal prognosis even in optimally treated patients. This is due to the two causes of resistance to current therapeutic modalities. The first is the process of bottleneck selection in the tumor mass or in its residues of those subclones, invariably present due to the complex and multifaced intratumor heterogeneity, with particular characteristics to resist and survive therapy which will emerge, expand and give rise to a new bulk. The second is the presence of quiescent founder CSCs which have micro-infiltrated the entire brain parenchyma at the very beginning of glioblastoma development or likely even before the growth of the primary tumor bulk. These founder CSCs have subsequently exited the cell cycle and settled dormant in G₀ state in the whole brain in perivascular parenchymal niches protected by their properties, the niche-microenvironment and the blood-brain barrier (Fig. 5).

Whenever possible, neurosurgical resection aimed at removing the entire bulk of the glioblastoma is recommend- ed. As mentioned, due to recent fundamental advances in neurosurgical strategies, many studies have proved that visible total removal of the primary bulk can be achieved in the majority of patients without compromising their functions (on average about 90% of cases nowadays). In these patients, without evident residual disease after surgery, the problem are those founder CSCs which have micro-infiltrated and conquered the entire brain parenchyma at the beginning of the glioblastoma's natural history and settle in specialized niches where they have exited the cell cycle and acquired the quiescent G₀ state. These cells represent a major challenge in developing new effective therapies. Indeed, as CSCs (the only cells which can survive for a long time in a dormant state in specialized niches) are the most resistant cells to all therapeutic modalities due to their particular properties (enhanced mechanisms of DNA repair, ability to extrude toxic agents, capacity to exit the cell cycle and resist apoptosis),16,86 Moreover, they are protected by the specific niche-milieu all around the brain where the blood-brain barrier is intact and can remain for a long time in the quiescent non-dividing state. As a consequence, they are not subjected to the effects of cytotoxic drugs which are effective against active replicating cells. For these reasons, recurrences inevitably happen after a certain period of time either in proximity or distantly to the previously operated bulk. This is due to the activation of the dormant parenchymal CSCs also in patients who underwent total removal of the primary tumor and current optimal postoperative treatment (i.e. conformational radiotherapy of the surgical cavity and concomitant and adjuvant chemotherapy).

In patients with residual disease after surgery, the situation is even more complicated and innovative therapies must take both causes of resistance into account. Indeed, with regards to the CSCs of the tumor mass, glioblastomas contain not only one specific CSC (i.e. the founder CSC) but multiple genetic and epigenetic CSC variants which have evolved during the trunk-branch progression of the tumor from the founder CSC. It follows that in these patients, in addition to the problem of the micro-infiltrated brain parenchyma, one or more different active residues containing multiple CSC variants (and their progeny) appear insuperable obstacles to current therapeutic regimens due to the establishment of rapid resistance. Indeed, at the beginning clinical therapy can decimate progenitors and committed cancer cells and even some sensitive subtypes of CSCs with a transitory reduction of the residual tumor burden. However, those variants of CSCs which possess particular characteristics to resist and survive therapy will emerge, expand via clonal selection and will inevitably re-form a tumor bulk (Fig. 5).

Strategies to follow to overcome resistance and find effective therapies against glioblastoma

From a neurosurgical point of view (being invariably the neurosurgical removal the first therapeutic option whenever possible), patients diagnosed with glioblastoma can be classified into three categories as follows: a) inoperable patients (butterfly or multifocal glioblastomas, sometimes in association with poor Karnofsky status) b) patients harboring a bulk that can be only partially removed (patients who have bulk tumor residual disease after surgery) c) patients harboring a bulk that can visibly be totally removed (patients who do not have evident bulk tumor residual disease after surgery)

a) Currently, for those patients diagnosed with inop- erable glioblastomas, like butterfly and or multifocal lesions, the prognosis is extremely poor. A neurosurgical neuronavigation-assisted procedure aimed at collecting a few samples for diagnosis purposes is the only option for the neurosurgeon. In these patients, the problems in thera- pies arise both from the heterogeneity of the bulk (or more bulks) containing multiple different genetic and epigenetic CSCs and from the micro-infiltrated brain parenchyma. The hope is that future treatments with proven effective-
cases (with the above mentioned exceptions where single contrast-enhancing and fluorescent bulk tumor. In these cases, despite excellent surgical efforts in neurosurgical techniques, whenever the glioblastoma is not very large and located within or near eloquent brain areas, has not been totally removed (residual fluorescence in some brain areas), one or likely more than one small heterogeneous different residues are left behind. It follows that the emergence of resistance to current therapeutic treatments due to the selection of the CSC subpopulations with the characteristics to resist therapy is inevitable for patients of the b group as well as for patients of the c group (not operated on).

Given the described scenario, two research areas should be attentively pursued to win this impasse. The first way to overcome the resistance of one or more different residues may be the finding of one (or more) particular modalities of self-renewing alternating phases of symmetrical and asymmetrical division to give rise to the bulk and the different proportions of CSCs during the stages of tumor development (in addition to external factors, e.g. hypoxia and the different replication time of progenitors and committed cells).

Another way may be the finding of one (or more) particular and exclusive marker of the founder CSC that can be exploited for therapy. Such a marker would be ubiquitously expressed in every CSC of the residues and it should be verified in future studies if it might be associated to a particular genetic alterations related to exclusive and stable (not bypassed by other mechanisms or mutations) CSC features or properties (not shared by normal stem cells) that can be exploited in developing new therapeutic solutions. In relation to this, genomic and molecular characterization of CSCs is of crucial importance because it can enable researchers to study and highlight those mechanisms related to self-renewal capacity and impaired asymmetrical division in CSCs. Indeed, CSCs must possess their own particular modalities of self-renewing alternating phases of symmetrical and asymmetrical division to give rise to the bulk and the different proportions of CSCs during the stages of tumor development (in addition to external factors, e.g. hypoxia and the different replication time of progenitors and committed cells). Another way may be the finding of one (or more) particular and exclusive marker of the founder CSC that can be exploited for therapy.

Figure 4. Recent genetic studies have confirmed that local recurrences develop from active residues (evident or not visible) left behind after glioblastoma bulk resection. Indeed, local recurrences share all the mutations happened in a sector of the previously removed (and phylogenetic reconstructed) bulk and in addition other genetic alterations due to their own evolution. On the contrary, distant recurrences share with their primary tumor only the founding or ancestral mutations (corresponding to the trunk) and do not possess the genetic alterations occurred during the bulk branched progression. In addition to the founding mutations, distant recurrences show new genetic alterations due to their own evolution.

As a consequence of what above, it appears evident that the therapeutic battle against glioblastoma and the search for future effective treatments must selectively target the CSC populations taking into account the two causes of resistance in relation to the three groups of glioblastoma patients (Fig. 5):

1. the bulk tumor (for patients classified in a and b groups)
2. the micro-infiltrated brain parenchyma (for patients classified in a and b groups as well as for patients of the c group)

1. Bulk tumor (patients of a and b group)

"With variants being continually produced, and even increasing in frequency with tumor progression, the neoplasm possesses a marked capacity for generating mutant sublines, resistant to whatever therapeutic modality the physician introduces." P.C. Nowell. The clonal evolution of tumor cell populations. Whenever the primary bulk, due mainly to its relationship with critical brain regions, has not been totally removed (residual fluorescence in some brain areas), one or likely more than one small heterogeneous different residues are left behind. It follows that the emergence of resistance to current therapeutic treatments due to the selection of the CSC subpopulations with the characteristics to resist therapy is inevitable for patients of the b group as well as for patients of the c group (not operated on).

Given the described scenario, two research areas should be attentively pursued to win this impasse. The first way to overcome the resistance of one or more different residues may be the finding of common CSC specific genomic alterations related to exclusive and stable (not bypassed by other mechanisms or mutations) CSC features or properties (not shared by normal stem cells) that can be exploited in developing new therapeutic solutions. In relation to this, genomic and molecular characterization of CSCs is of crucial importance because it can enable researchers to study and highlight those mechanisms related to self-renewal capacity and impaired asymmetrical division in CSCs.

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driver lesion of the founding clone, without pre-existing resistant mutations in minority subclones and or not bypassed by alternative mechanisms or pathways at treatment introduction, or be the one(s) used early for invasion and conquest of the micro-infiltrated brain parenchyma (see below). This marker must be exclusive of CSCs and not shared by normal stem cells.

2) Micro-infiltrated brain parenchyma (patients of a, b and c groups)

Radiological, clinical and surgical clues, recent evidence from distant recurrences and similarities with the metastatic CSCs in other tumor types\(^1\) indicate that the brain of patients with glioblastoma is micro-infiltrated by quiescent long-surviving CSCs (pre-hypoxic) settled in a dormant \(G_0\) state in perivascular parenchymal niches of the “healthy” brain parenchyma (where the blood-brain barrier is intact). Due to what previously mentioned, it appears plausible that in the parenchymal niches also (or better still exclusively, see below) CSCs of the trunk (founder CSCs) will be found. Thus, future studies aimed at identification and characterization of the quiescent CSCs in the micro-infiltrated brain parenchyma as well as of one or more exclusive marker(s) used by them to settle dormant (involving mechanisms of adhesion, anchorage and cell cycle arrest) in the perivascular parenchymal niches will be of fundamental importance. Currently, we do not have direct experimental evidence to demonstrate if all the CSCs settled dormant in the brain parenchyma come only from the first wave of invasion (performed by the founder CSCs) or if there are subsequent waves of infiltration and settlement in the niches during bulk growth and progression. Obviously, the CSCs of the branches retain the ubiquitous genetic alterations of the founder CSC, but epigenetic and molecular variations due to the hypoxic phases and restriction of resources during tumor development can limit their migratory capacity while enhancing at the same time their ability to resist hypoxia and nutrient deprivation.\(^34\) Importantly, from this we could also deduce that the key differences in migratory capacity and settlement in a different niche between the normal cell of origin and the founder CSC are likely to be of epigenetic and or transcriptomic nature. Before direct experimental verification, we already have some clues about the CSC waves of infiltration by the indirect way to study the micro-infiltrated brain parenchyma through the analysis of distant recurrences. As mentioned, the majority of glioblastoma recurrences happen locally due to rapid emergence and development of active CSCs left behind after neurosurgical resection which have resisted radio-chemotherapy. In these cases, the genetic analysis of the recurrence should show (and indeed it does) many similarities with one or more samples (corresponding to the bulk region from which the recurrence originates) of the primary tumor. On the contrary, distant recurrences should emerge due to activation of quiescent founder CSCs in the parenchymal niches in cases of previous complete removal of the primary bulk (group c patients). Indeed, if distant recurrences are due to only one or few early waves of invasion, they should be genetically very different to many samples of the bulk corresponding to middle and late stages of development (or to the branches of the tree) and show affinities with the founding clone (the trunk of the reconstructed phylogenetic tree) otherwise they would be genetically similar to local recurrences. The results that have come from two recent studies\(^59,60\) are providing the first indirect proof to confirm these concepts. Indeed, in these studies, the pattern of distant recurrences shares the ancestral mutations of the founding clone, lacks the middle and late phases of bulk development and possesses new mutations due to their own evolution. This indicates and provides indirect experimental evidence that the cell (or better still the CSC) giving rise to the distant recurrences is one identical to the cell which originated the trunk of the bulk. Therefore, we can deduce that the cell which has the features and the capacity to originate distant recurrences, migrated and micro-infiltrated the whole brain parenchyma during or likely before the trunk tumor development and not during the branched progression of the primary bulk. Thus, in the case of primary glioblastoma the micro-infiltration of the brain parenchyma should travel in parallel or even precede the bulk tumor growth and be per-

![Figure 5. Two are the causes of resistance to current therapeutic treatments: (1) The entire bulk(s) (in patients not operated on) or its residues after subtotal surgical resection contain multiple CSC variants. After post-therapeutic temporary regression, the CSC subpopulations with particular features to resist therapy will be selected and will invariably emerge, expand and reform the tumor. (2) The activation of the diffusely micro-infiltrated quiescent CSCs leads to the emergence of one or more distant recurrences even when the primary bulk was of a relatively small size and unambiguously totally removed (e.g. currently by polectomy or lobectomy or in the past by right hemispherectomy).](image-url)
It is now clear that in order to finally find a solution to this devastating disease, new treatment modalities must consider both the acquisition of resistance by different active CSCs in the primary bulk(s) or in its residues after surgery and the silent presence of founder CSCs in the whole brain parenchyma protected by their quiescent status, the niche-microenvironment and the blood-brain barrier.

Given what mentioned, the ways proposed in this paper to study the tumor bulk and the micro-infiltrated brain parenchyma and those to find novel and effective therapies can hopefully overcome a current insuperable impasse and open a new era in the treatment of glioblastoma patients.

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Уникальные свойства глиобластомы определяют направление инновационных методов терапии в эпоху эволюционной модели разветвлённого дерева

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РЕЗЮМЕ

Мультиформная глиобластома представляет собой солидную опухоль с особыми свойствами, обусловленными органом, в котором она появляется и разными проявлениями её развития. Мозг исключительно чувствителен к нехватке кислорода и глюкозы и является единственным органом, который не может быть трансплантирован или полностью удален. Кроме того, многие предпосылки и недавние косвенные экспериментальные данные указывают на то, что микроинфильтрация всей паренхимы головного мозга происходит на очень ранних стадиях роста опухоли или, вероятно, даже до этого. Как следствие, первичная глиобластома (IDH - wildtype, WHO 2016) является единственной опухолью, при которой злокачественные (с отдалённой инфильтрацией органа появления) и смертельные (основная причина смерти пациента) фазы совпадают и накладываются в одной единственной фазе её естественного развития. На сегодняшний день прогноз для оптимально пролеченных пациентов с глиобластомой продолжает быть безнадежным, несмотря на нынешний фундаментальный прогресс в нейрохирургических методах, которые позволяют улучшить максимально безопасную резекцию и выживаемость. Внутриопухолевая неоднородность объёма глиобластомы, являющаяся следствием эволюционной модели разветвлённого дерева, очень ранняя микроинфильтрация и расположение неопластических клеток во всей паренхиме головного мозга являются причинами резистентности к современным методам терапии. С целью развития будущих инновационных и эффективных методов лечения в данной статье рассматриваются уникальные свойства глиобластомы, соответствующие методы исследования, а также стратегии, которым необходимо следовать для преодоления настоящих причин резистентности к терапии.

Ключевые слова:

уникальные свойства глиобластомы, эпоха эволюционной модели разветвлённого дерева, исследовательские методы, причины резистентности, инновационные методы терапии