Key concepts in glioblastoma therapy

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ABSTRACT

Glioblastoma is the most common form of primary brain cancer and remains one of the most aggressive forms of human cancer. Current standard of care involves maximal surgical resection followed by concurrent therapy with radiation and the DNA alkylation agent temozolomide. Despite this aggressive regimen, the median survival remains approximately 14 months. Meaningful strategies for therapeutic intervention are desperately needed. Development of such strategies will require an understanding of the therapeutic concepts that have evolved over the past three decades. This article reviews the key principles that drive the formulation of therapeutic strategies in glioblastoma. Specifically, the concepts of tumour heterogeneity, oncogene addiction, non-oncogene addiction, tumour initiating cells, tumour microenvironment, non-coding sequences and DNA damage response will be reviewed.

INTRODUCTION

Glioblastoma is the most common form of primary brain tumour. The incidence of this tumour is fairly low, with two to three cases per 100,000 people in Europe and North America. It is one of the most aggressive forms of human cancer. Without treatment, the median survival is approximately 3 months. The current standard of treatment involves maximal surgical resection followed by concurrent radiation therapy and chemotherapy with the DNA alkylation agent temozolomide (TMZ). With this regimen, the median survival is approximately 14 months. For nearly all affected, the treatment remains palliative.

The best available evidence suggests that glioblastomas originate from cells that give rise to glial cells. These glial-derived tumours are graded by WHO into four categories, termed WHO grade 1–4. The higher grade denotes histological features of increased malignancy. WHO grade 4 glioma is essentially synonymous with glioblastoma.

Studies carried out over the past three decades suggest that glioblastomas, like other cancers, arise secondary to the accumulation of genetic alterations. These alterations can take the form of epigenetic modifications, point mutations, translocations, amplifications or deletions and modify gene functions in ways that deregulate cellular signalling pathways leading to the cancer phenotype. The exact number and nature of genetic alterations and deregulated signalling pathways required for tumorigenesis remains an issue of debate, although it is now clear that central nervous system (CNS) carcinogenesis requires multiple disruptions to the normal cellular circuitry. These genetic alterations result in either activation or inactivation of specific gene functions that contribute to the process of carcinogenesis. Genes that, when activated, contribute to the carcinogenesis are generally termed proto-oncogenes. The mutated forms of these genes are referred to as oncogenes. Genes that, when inactivated, contribute to the carcinogenesis are termed tumour suppressor genes.

Recent research in the area of experimental and clinical oncology has identified the key signalling pathways, critical regulatory nodes, genes and their protein products, and their mutual cross-talks, thereby providing a solid molecular basis for selection of candidate therapeutic targets and drug discovery programmes. These lines of investigation complement the recent efforts to sequence entire genomes of a growing number of human tumours including glioblastoma. The efforts have led to the formulation of new concepts and principles in tumour cell biology. Exploitation of these major advances has begun to provide exciting leads that may afford innovative therapeutic strategies. This article will aim to review these critical concepts and their relevance for glioblastoma therapeutic development.

CONCEPT 1: Glioblastoma Subtypes

There is an old adage that cancer is a hundred diseases masquerading in one. While this adage is based on clinical and pathologic observations, systemic genomic characterisation of a large number of glioblastoma specimens confirms the notion that subtypes with distinct pathological molecular events and therapeutic responses exist. The Cancer Genome Atlas project (TCGA) is a major National Institutes of Health initiative involving institutions spanning the continental USA with the goal of tumour specimen collection and molecular characterisation. Glioblastoma was one of the first tumour types characterised in this effort. This vast wealth of data is unprecedented, and despite the enormous challenge to process and analyse this incoming information, correlations of such emerging ‘genetic and expression profiles’ or ‘tumour landscapes’ with tumour biology and clinico-pathological features of the patients (including therapeutic responses) are beginning to impact oncology. These studies have led to the understanding of glioblastoma as an umbrella term that encapsulates subtypes characterised by distinct molecular properties. Based on global transcript profiling, glioblastoma can be divided into three to four distinct...
subtypes. The recognition that glioblastoma consists of subtypes varying in molecular circuitry and biological behaviour suggests that no therapy can be universally efficacious. The major importance of this concept of heterogeneity is that meaningful therapeutic gain can only be attained by customising the therapy to the underlying molecular circuit. One subtype (termed classical by the TCGA and proliferative by Phillips et al) is characterised by frequent amplification or mutations in the epidermal growth factor receptor (EGFR) gene. In contrast, another subtype, termed proneural by both groups, harbours frequent mutations in p53, platelet-derived growth factor receptor A and isocitrate dehydrogenase 1. A third type, termed mesenchymal, is characterised by frequent mutations in the neurofibromatosis type 1 (NF-1) gene.

Importantly, these transcriptional subtypes appear to differ in their clinical courses and therapeutic responses. In terms of prognosis, studies by Phillips et al and Verhaak et al demonstrated increased overall survival in patients with proneural glioblastoma relative to other molecular subtypes. In terms of therapeutic response, Verhaak et al explored this issue by stratifying the patients with various molecular subtypes into two groups: those that received concurrent chemo-radiation therapy or received more than three cycles of chemotherapy; and those that did not receive concurrent chemo-radiation therapy or received less than four cycles of chemotherapy. When stratified this way, the authors found that the two groups exhibited comparable survival in the proneural group. In contrast, for other molecular subtypes, patients in the first group exhibited improved survival relative to the second group. Since the analysis combined the survival effect of concurrent chemo-radiation therapy and prolonged chemotherapy, it is difficult to assess whether the effect is due to the former or the latter. Taken as a whole, these datasets suggest that the patients with the proneural glioblastomas tend to survive longer but are less responsive to conventional chemotherapy or chemo-radiation therapy (figure 1).

CONCEPT 2: ONCOGENE ADDICTION

The term ‘oncogene addiction’ was initially coined by Dr Bernard Weinstein to describe the phenomenon that some tumours exhibit exquisite dependence on a single oncogenic protein (or pathway) for sustaining growth and proliferation. Such dependence has been convincingly demonstrated in both tissue culture and transgenic mouse systems for oncogenic versions of MYC and RAS. Application of this concept to the clinical setting has achieved variable success in some cancer types, including chronic myelogenous leukaemia harbouring the BCR-ABL translocation, Erb2 overexpressing breast cancer, and non-small cell lung cancer harbouring a subset of EGFR mutations.

A simplistic application of this concept in glioblastoma would involve identification of the critical ‘addicted’ oncogene followed by the inhibition of such oncogenes. Unfortunately, the actual biology of glioblastoma is far more complex.

To understand this complexity, a careful analysis of the fundamental notion of oncogenic addiction is needed. In some ways, the observation that tumours exhibit dependence on a particular oncogenic pathway at some point in its history is not surprising. However, considering the plethora of dynamic genetic changes that accumulated during cancer progression, it is somewhat counter-intuitive to suspect that any particular pathway would play a prominent role in maintaining cell viability. Moreover, inactivation of the normal counterpart of the addicted oncogenic protein is often tolerated in normal tissue. These observations suggest that the genetic circuitry of the cancer cell have been extensively reprogrammed to result in this ‘addicted’ state.

The molecular nature of this reprogramming remains poorly understood. Several hypotheses have been put forward. One hypothesis involves the notion of ‘genetic streamlining’, where genetic instability in cancer cells is thought to mutagenically or epigenetically inactivate certain signalling pathways that are operational in a normal cell but not required for growth in the cancer cell. In this ‘streamlined’ state, the tumour cell becomes hyper-dependent on the oncogene-driven processes. A more generalised form of this explanation involves the notion of synthetic lethality. Two genes are considered synthetically lethal if cells remain viable with inactivation of either gene. Simultaneous inactivation of both genes, on the other hand, results in cell death. It is thought that the cancer cells have accumulated mutations that are synthetically lethal with the absence of critical oncogenes. The main difference between this hypothesis and the ‘streamline’ hypothesis is that the mutation in the former can result in a gain or loss of function, whereas the latter specifically proposes a loss of function. A third hypothesis suggests that oncogenes reprogramme the tumour cell by both pro-survival and pro-apoptotic signalling. With acute inactivation, the pro-survival signalling decayed faster than the pro-apoptotic signalling, resulting in tumour death. This thesis has been coined the ‘oncogene shock’ hypothesis.

The main reason for revisiting the framework of oncogene addiction is to discuss the mechanism by which the cells can evolve to avoid such addiction. For instance, in the context of synthetic lethality, EGFR inhibition may be cytotoxic to glioblastoma cells only in the appropriate genetic context. Indeed, therapeutic effects of EGFR inhibition were observed only in patients with tumours expressing an oncogenic form of EGFR and an intact phosphatase and tensin homolog (PTEN) tumour suppressor gene. To complicate the matter, recent studies demonstrate that glioblastomas harbour activation of multiple oncogenic receptor tyrosine kinases, such that inactivation of any single oncogene merely diverts signalling through other active oncogenes. In these contexts, it is evident that meaningful therapy will require simultaneous inhibition of multiple oncogenes or identification of the fitting genetic context.

CONCEPT 3: NON-ONCOGENE ADDICTION

Emerging literature suggests an alternative strategy to the multitarget approach. These studies reveal that oncogene activation introduces secondary physiological changes that stress cellular

Figure 1 Transcriptional subtypes of glioblastoma.
capacity for survival. Consequently, tumour cells becomes more dependent (or hyper-dependent) on processes required to compensate for these stressful conditions. This phenomenon is termed ‘non-oncogene addiction’ since the compensatory processes required for tumour survival do not directly contribute to the cancer formation. In other words, even the genes that are not themselves targeted by tumorigenic mutations may well become essential for the tumour to survive the stressful environment and fuel the demanding process of tumour progression. Consequently, interfering with the function of such genes could cause tumour kill while sparing the normal counterpart (figure 2).

There are several examples of such critical non-oncogenic pro-survival functions required for the maintenance of the tumorigenic state in glioblastoma. EGFR is a critical proto-oncogene in glioblastoma pathogenesis. Our laboratory has demonstrated that EGFR hyperactivation results in an increased accumulation of reactive oxygen species (ROS), which in turn cause cytotoxic DNA damage. To compensate for the deleterious effect of ROS, EGFR hyperactive glioblastomas exhibit increased reliance on the DNA repair process required for the repair of ROS-related DNA damage. Selective targeting of EGFR hyperactive glioblastomas can, thus, be achieved by inhibition of these repair processes. Other groups have demonstrated that EGFR hyperactivation in glioblastoma cell lines heightens requirement for lipogenesis.

Additional examples of such critical non-oncogenic pro-survival functions required for maintenance of the tumorigenic state include dependency on mechanism for compensating mitotic and proteotoxic stress and interplay with the tumour microenvironment including the immune system.

The principle of non-oncogene addiction suggests that there is a wider spectrum of therapeutic options than afforded under the paradigm of ‘oncogene addiction’. In many cases, compensatory processes involved in ‘non-oncogene addiction’ are the same as those that basic scientists have studied for years (for instance, DNA repair). Mechanistic investigations into these biological processes by the basic scientists have yielded a rich database of inhibitors. Thus, identifying gene functions that compensate for oncogene-induced cellular stress should afford opportunities to tap into this rich database and expand the denominator of drugs available for combinatorial therapy. Targeting genes that are synthetically lethal with oncogenes constitutes an attractive means to this end.

It is important to note that the effects of therapies designed based on the principles of ‘oncogene addiction’ and ‘non-oncogene addiction’ are inherently antagonistic. For instance, EGFR inhibition leads to a reduction in ROS, obviating the need for DNA repair. In this context, the combination of DNA repair inhibition and EGFR inhibition would not be desirable. Rational strategies for synthesising the two therapeutic paradigms remains a major intellectual challenge.

CONCEPT 4: TUMOUR-INITIATING CELLS

Another advance that may profoundly change our thinking about solid tumours including glioblastoma involves the concept of tumour-initiating cells. The experimental observation is that within a total population of glioblastoma cells, there appears to be a small subpopulation of cells that are highly tumorigenic (hence the term ‘tumour-initiating cells’ or ‘TICs’), with capacity for self-renewal. In some studies in which severely immune-compromised mice are used as assay for melanoma xenograft formation, the proportion of TICs within a tumour has been reported to be as high as 27%. Because glioblastoma TICs share many common properties with neural stem cells, it is proposed that TICs originated from stem cells. While there are some data supporting this hypothesis, the universality of this hypothesis remains controversial.

Protein markers to prospectively identify and isolate these putative TICs have been reported, such as the transmembrane glycoprotein CD133 (prominin-1) in glioblastomas. However, the value of CD133 as a single marker of glioblastoma TICs remains controversial, partly because CD133-negative glioblastoma cells could also give rise to tumours in a intracranial xenograft model. These uncertainties motivate an ongoing search for additional candidate TIC markers. Candidate cell surface molecules suggested in this context include the adhesion glycoprotein L1CAM, surface carbohydrate antigen CD15 (SSEA-1), surface marker A2B5 and integrin α6. Currently, there are no generally accepted cell surface markers for defining TICs. The definition of TICs remains a functional one as defined by the ability of a tumour cell to sustain self-renewal and initiate glioblastoma formation in immunocompromised xenograft models.

Arguably, the most important aspect of the concept of TICs is that this population appeared particularly resistant to conventional radiation and chemotherapy. In this context, TICs may be responsible for glioblastoma recurrence after conventional therapy. Given such properties, it is understandable that glioblastoma research has recently focused on identification and development of potential anti-TIC therapies. Two of these strategies, namely targeting the TICs as part of a vascular niche, and attempts to overcome their therapeutic resistance, are discussed in the following sections on glioblastoma angiogenesis and the role of DNA damage response pathways, respectively. Here, we briefly consider other strategies that are emerging as potentially fruitful approaches to treat glioblastoma through targeting TICs.

The first strategy reflects the efforts to identify suitable cell surface markers to reliably identify glioblastoma TICs—with the hope of conjugating the corresponding antibody to cytotoxic compounds as therapeutic agents. The second strategy is based...
on observations that some TICs, like neural stem cells, can be induced into a differentiated state in which the self-renewal properties are lost. Among the suggested agents to induce such TIC differentiation, the bone morphogenetic proteins appear promising. The third strategy involves modulating specific signalling pathways required for maintaining the TIC state. Pathways targeted include those mediated by EGFR, Wnt-beta catenin, signal transducer and activator of transcription 3, Sonic Hedgehog-Gli and Notch pathways. Finally, normal neural stem cells have been shown to migrate towards and track TICs. Based on this principle, neural stem cells have been used as delivery vehicles to increase local concentration of therapeutic agents in the vicinity of TICs.

CONCEPT 5: TUMOUR MICROENVIRONMENT

Over the past two decades, conceptualisation of glioblastomas has evolved from a collection of relatively homogenous cells to the recognition of distinct subpopulations of tumour cells to that of a complex organ, with constant interactions between tumour cells and aberrant stromal elements. Analogous to the distinct functions of different tissues in an organ, genomic characterisation using cells derived from distinct regions of the tumour revealed genetic heterogeneity. A major concept in oncology has emerged that reciprocal signalling between the distinct subpopulations of neoplastic cells and the aberrant stromal elements serve to sustain progressive neoplastic transformation and possibly functional specialisation. Understanding of these interactions has afforded novel therapeutic targets. For the purpose of this review, distinct subpopulation of neoplastic cells (tumour heterogeneity) and aberrant stromal interactions will both be considered as components of the microenvironment.

Studies of EGFR revealed a beautiful illustration of signalling between the subpopulations of genetically distinct neoplastic cells in glioblastoma. EGFRvIII is a variant of EGFR that arose from spontaneous deletion of exons 2–7. This variant is present in about 20% of glioblastomas and results in constitutive hyper-activation of EGFR. Clinical studies suggest that patients with glioblastoma harbouring this variant tend to have a worse prognosis. Interestingly, the vIII variant is rarely found in the absence of EGFR overexpression. Further, when found, the variant is typically present in only a subset of the total tumour mass. Investigations into the molecular mechanism underlying these observations revealed that EGFR-vIII overexpression increased the secretion of interleukin 6 (IL-6) and leukaemia inhibitory factor, two soluble cytokines. These cytokines trigger phosphorylation of gp130 in the non-EGFRvIII expressing cells, which in turn activate EGFR of these cells. This activation increases the tumorigenicity and aggressiveness of the cancer. Such signalling may serve to actively maintain tumour cell heterogeneity (figure 4).

In addition to the signalling between distinct and genetically defined subpopulations of tumour cells, normal cells without genetic alterations associated with carcinogenesis are often recruited to the foci of tumour cells. In the process, these normal cells undergo phenotypic changes in response to direct physical interaction with cell surface proteins on the tumour cells or through interaction with secreted soluble factors. These changes result in the release of growth factors that further enable and sustain neoplastic transformation or lead to new blood vessel formation. Cycles of such reciprocal interaction facilitate stepwise progression in neoplastic progression.

In terms of the non-neoplastic cell types shown to facilitate neoplastic information, they can generally be divided into three categories. The first category involves endothelial cells or endothelial cell precursors. These cells are critical for tumour growth since there are inherent limitations on the distance that oxygen and macromolecules can travel. In xenograft models, solid tumours can only proliferate up to a size of 1–2 mm without the development of a new blood supply. Quiescent endothelial cells in proximity of the neoplastic foci may be induced to initiate biological programmes that lead to blood vessel formation by secreted factors such as vascular endothelial growth factor (VEGF, see below). Alternatively, endothelial cell precursors in the bloodstream may be recruited into the tumour foci. A final mechanism involves the trans-differentiation of TICs to become endothelial cells.

The second class of non-neoplastic cells that actively participate in tumour progression is fibroblasts. There is good evidence that these otherwise genetically normal fibroblasts, when in proximity of tumour cells, can become ‘re-programmed’ to promote/sustain neoplastic transformation. Transplantation experiments mixing cancer-associated fibroblast with cancer cells lead to a more aggressive tumour phenotype than ‘normal’ tumour cells. This tumour-promoting activity is largely thought to be the combined effect of cell-to-cell interaction and cytokine release. Non-neoplastic astrocytes perform many of the functions associated with fibroblasts. Thus, the interactions between glioblastoma cells and non-neoplastic astrocytes warrant further investigations.

The final class of non-neoplastic cells recruited are cells that mediate immune function. In general, these cells may possess tumour-antagonising activity or tumour-promoting activity. These divergent properties may be rationalised by understanding that the immune system is required for both the destruction of foreign cells and facilitating wound healing. Properties associated with the former will likely lead to tumour ablation. However, cytokines and growth factors associated with wound healing may promote tumour growth.

The glioblastoma cells have evolved a large number of mechanisms that allow escape from immune detection and ablation, including release of immunosuppressive cytokines, such as IL-10, cytotoxic T-lymphocyte antigen 4 and transforming growth factor β, expression of cell surface molecules that facilitate immunosuppression, such as B7-H1. These events, in turn, lead to the induction of regulatory T cells (Treg), downmodulation of antigen-presenting cells, with concomitant loss of T-cell effector function or loss of functional major histocompatibility class I receptors.

These factors contribute to a ‘hostile’ microenvironment that compromises the immune cells’ ability to achieve tumour eradication. For instance, primed CD8+ cytotoxic T cells can
penetrate the blood—brain barrier and access the CNS.\textsuperscript{70} However, they are incapable of tumour eradication. Indeed, in patients with glioblastoma, tumour progression is seen despite the presence of tumour-infiltrating lymphocytes.\textsuperscript{71}

Interestingly, glioblastoma’s capacity to suppress immune response appears intimately associated with the process of neoplastic transformation. PTEN encodes a tumour-suppressing phosphatase that is frequently mutated during glioblastoma pathogenesis. The translation of many immune-suppressive cytokines and molecules, including IL10 and B7-H1, are under the regulation of PTEN. Thus, PTEN loss during neoplastic transformation leads to increased expression of immune-suppressive cytokines and cell surface molecules.\textsuperscript{67} This expression, in turn, creates a hostile environment for immune cells that otherwise target tumour for ablation.

Understanding the interaction between the genetically distinct subpopulation of glioblastoma cells and their microenvironment has yielded novel therapeutic developments. The endeavour most frequently cited in this regard involves angiogenesis inhibitors. Realising that VEGF is critical in angiogenesis, bevacizumab, a humanised antibody against VEGF, was developed.\textsuperscript{72} While there has not been a randomised control trial to assess the efficacy of bevacizumab, a phase II clinical trial demonstrated improved progression-free survival in recurrent glioblastomas (after concurrent TMZ/radiation treatment) relative to historical data of patients who received TMZ at recurrence.\textsuperscript{73–75} However, no overall survival benefit has been demonstrated with bevacizumab treatment. Clearly, angiogenesis inhibition is but one of the many strategies that can be developed based on the concept of tumour microenvironment.

CONCEPT 6: NON-CODING DNA SEQUENCES

Classically, coding sequences are defined as the strand of DNA that has the same base sequence as the RNA transcript produced (with the caveat that thymines are replaced by uracil) that are ultimately translated into proteins. While the identification of nucleotide alterations within the coding sequences of proto-oncogene or tumour suppressor genes has significantly contributed to our understanding of carcinogenesis, there is an emerging appreciation that alterations in non-coding sequences similarly contribute to carcinogenesis.\textsuperscript{76} A notable example involves the regulation of gene transcription by reversible modification of gene promoter regions—a phenomenon sometimes referred to as ‘epigenetic regulation’.\textsuperscript{77} Similarly, we are beginning to appreciate the importance of transcripts that do not encode for proteins but are transcribed, such as microRNAs (miRNAs)\textsuperscript{78} and long non-coding RNAs (or lincRNAs),\textsuperscript{79,80} in terms of transcriptional and post-transcriptional modifications. The concept that non-coding DNA sequences regulate gene function and impact carcinogenesis has significantly expanded the repertoire of strategies available for glioblastoma therapeutics. To review this concept, we will discuss illustrative examples of epigenetic regulation, miRNAs, and lincRNAs (figure 5).

The term ‘epigenetic regulation’ has been coined to describe the phenomenon that heritable changes in gene expression can occur in the absence of changes in the DNA sequences encoding for gene function.\textsuperscript{77} The mechanism underlying this regulation involves cytosine methylation\textsuperscript{81} or histone modifications that, in turn, modulate the accessibility of gene promoter regions to transcriptional factors.\textsuperscript{82} Cytosine methylation typically occurs in the context of CpG di-nucleotide repeats, or CpG islands.\textsuperscript{83} Promoters harbouring heavily methylated CpG islands are typically transcriptionally silenced. There are two types of promoter methylation that are particularly pertinent to glioblastoma therapy: methylation in the promoter region of the DNA repair gene, methyl-guanine methyl transferase (MGMT) and the glioma-CpG island methylator (G-CIMP) phenotype. MGMT encodes an enzyme that removes alkyl adducts at the O6 position of guanine.\textsuperscript{83} Because alkyl modification at this position is highly toxic and constitutes the primary mechanism for the tumoricidal activity of the chemotherapeutic agent TMZ, MGMT expression level correlates well with TMZ response in patients with glioblastoma.\textsuperscript{84} The human MGMT gene possesses a CpG island that spans approximately 1000 bases around the transcriptional start site. Detailed analysis of this region revealed 108 CpG sites\textsuperscript{85} that are methylated. Methylation of a subset of these CpGs has been associated with transcriptional silencing of MGMT\textsuperscript{86,87} and is associated with improved clinical outcome in patients with glioblastoma receiving TMZ therapy. Interestingly, MGMT promoter methylation is also associated with improved survival in patients who did not receive TMZ therapy.\textsuperscript{88,89} While the mechanism underlying this observation remains unclear, it seems likely that MGMT may participate in detoxifying the accumulation of endogenous DNA damage that is typically associated with the oncogenic state.\textsuperscript{82} As discussed in concept 7, glioblastoma cells accumulate endogenous DNA damage in the absence of DNA damaging agents.\textsuperscript{82} These endogenous DNA damages are not unlike those induced by temozolomide or radiation in that they could trigger cell death if unrepaired. Thus, tumours with high levels of MGMT may grow more robustly since MGMT is capable of detoxifying these endogenous DNA damages. If the tumour cells grow more robustly, the patient will survive for a shorter duration. In contrast, the glioblastoma cells with low MGMT may be more susceptible to the deleterious effects of the endogenous DNA damages. These tumours may grow less robustly, resulting in longer patient survival.

The G-CIMP phenotype refers to the observation that a subset of glioblastomas exhibits concerted CpG island methylation at a large number of loci.\textsuperscript{90} Since genes required for tumour growth are located at many of these loci, glioblastomas harbouring the G-CIMP phenotype tend to be more benign. Correspondingly, patients with G-CIMP glioblastomas experienced significantly improved outcome. Understanding the concept that the patterns of CpG island methylation directly impact outcomes in patients with glioblastoma open the door to therapeutic strategies aimed at enhancing promoter methylation at select promoter loci. Importantly, recent studies suggest that promoter methylation at distinct loci may be affected by specific chromatin-modulating factors.\textsuperscript{91}

miRNAs are small non-coding RNAs of 20–22 nucleotides that, through imperfect pairing, bind to the 3’ untranslated regions of protein-coding mRNAs. Typically, this binding leads to mRNA degradation or inhibition of protein translation to suppress the expression of the target proteins.\textsuperscript{78} Bioinformatic analysis predicts that a single miRNA can potentially regulate hundreds of target oncogenes or tumour suppressor proteins. Expectedly, miRNAs have been implicated in carcinogenesis and
resistance to chemotherapy. As one illustrative example, our laboratory recently demonstrated that the protein MGMT is under the regulation of miR-181d. Cell biological studies revealed that binding of miR-181d to the 3′ untranslated regions of MGMT caused decreased MGMT expression. This inverse relationship was validated in glioblastoma specimens. Importantly, patients with high miR-181d expression (hence low MGMT) are more likely to respond to TMZ chemotherapy.

LincRNAs are transcripts >5 kb that are evolutionarily conserved across mammalian genomes. These RNAs are transcribed by polymerase II but do not encode proteins. The LincRNAs serve to suppress transcription by targeting chromatin-modifying complexes to specific genomic loci. While the role of LincRNA in glioblastoma awaits careful scrutiny, LincRNA have been shown to mediate the function of tumour suppressor genes pertinent to glioblastoma pathogenesis. As one example, TP53 encodes a transcription factor that regulates gene sets critical for cell cycle progression and apoptosis. Under normal conditions, p53 is a short-lived protein. In response to cellular stress (for instance, DNA damage or oncogene expression), p53 undergoes post-translational modifications and protein–protein interactions that enhance its stability and transcriptional activity. One of the downstream effectors of p55 is a LincRNA. This LincRNA serves as a key mediator to suppress transcription of other p53 effectors. Such mechanisms may be operational in glioblastomas.

Understanding the concept that non-coding sequences play critical roles in glioblastoma pathogenesis and resistance to chemotherapy offers novel strategies for biomarker development and therapy. For instance, direct introduction of select miRNAs into glioblastoma has been shown to inhibit growth and proliferation. Similarly, incorporation of miR-181d expression level may further augment the predictive value of MGMT promoter methylation. Importantly, the concept predicts certain situations where the effects of oncogenic mutation can be avoided by the effects of non-coding sequences. Integrating the biology of non-coding sequences in the context of mutational profile will be critical in understanding tumour physiology and meaningful therapeutic development.

**CONCEPT 7: DNA DAMAGE RESPONSE**

From a broader perspective, the status of the molecular machinery that detects, signals and repairs DNA damage, and overall orchestrates the multifaceted cellular response to genotoxic insults (here referred to as the DNA damage response: DDR) critically impacts tumour development and clinical outcome. While this is arguably relevant for any type of tumour to some extent, the DDR concept is particularly important for glioblastomas for the following reasons. First, the standard-of-care non-surgical modalities used to treat glioblastomas, namely ionising radiation and TMZ-based chemotherapy, operate through their genotoxic effects by causing mainly DNA double strand breaks (DSBs) and alkylated DNA lesions, respectively. Therefore, each individual patient’s germ-line disposition of the DDR-related genes, along with any somatic alternations within the DDR machinery that have been selectively acquired by the tumour dictate (along with other factors such as the tumour microenvironment discussed above) their response to therapy. Second, among the hallmarks of glioblastomas is their resistance to radiotherapy and chemotherapy. These phenomena highlight the intimate involvement of the cellular DDR network, particularly DNA damage signalling, cell-cycle checkpoints and DNA repair pathways, in the pathobiology of glioblastomas. Third, the harmful side effects of the standard therapies, including brain damage and consequently cognitive changes, are also attributable to DNA damage and the cellular and tissue responses to such treatments. Fourth, genetic and/or epigenetic aberrations of a range of DDR factors, including the above mentioned p53 tumour suppressor or DNA repair genes such as MGMT, occur commonly during glioblastoma pathogenesis and/or upon treatment. This aspect of gliomagenesis has been suspected and partly known for years, however it has only been validated by the recent insights gained through comprehensive analyses by complete tumour genome sequencing within the framework of the TCGA initiative. Finally, the TICs (see concept 4), appear to be particularly resistant to DNA-damaging therapies. This resistance is, at least in part, due to enhanced DNA damage signalling and checkpoint machinery.

Conceptually very relevant for such DDR-related features of gliomas is the recently described strong, constitutive activation of the DDR signalling pathways, observed from the early stages (grade II gliomas) of gliomagenesis up to glioblastomas. This spontaneous DDR activation precedes any genotoxic treatment, and it appears to be even more pronounced in gliomagenesis than in early lesions of major epithelial tumour types, in which this phenomenon represents a candidate intrinsic barrier against activated oncogenes and tumour progression. A major source of such DDR activation in early lesions including low-grade gliomas appears to be oncogene-induced replication stress, while in later stages of tumour progression, particularly in glioblastomas, the constitutive DNA damage signalling is fuelled by continued replication stress and by enhanced oxidative stress. Biologically, such oncogene-evoked DDR activation often leads to cell death or permanent proliferation arrest known as cellular senescence. This activation eliminates nascent tumour cells from the proliferative pool, thereby delaying or preventing tumour progression. Those lesions that do progress in the face of such constitutively activated DDR often do so by selection of various defects along the DDR signalling or effector pathways, such as mutations in the ATM-Chk1-Chk2-p53 DDR pathway. Importantly, while such selected DDR aberrations facilitate tumour progression by allowing escape from DDR-induced senescence or apoptosis, the very same defects may create tumour-specific vulnerabilities that can be exploited by therapeutic strategies based on the synthetic lethality principle (see concept 2 above).

In terms of exploiting the status of the DDR machinery for glioblastoma therapies, two major avenues are under intensive research and validation. First, there are promising attempts to sensitise glioblastoma cells (including the more resistant TICs) to conventional genotoxic therapy, such as ionising radiation, by concomitantly inhibiting the DNA damage signalling to downstream checkpoint and repair effectors. This strategy relies mainly on small molecule inhibitors of DDR kinases ATM, ATR, Chk1 and Chk2. This strategy appears particularly suitable for tumours with mutant p53. Such cancer cells lack the major p53-dependent G1/S checkpoint, and upon inhibition of the DDR kinases (whose activity underlies the still operational G2/M checkpoint) enter mitosis with an overload of unrepaired DNA damage, both endogenous and therapy induced, followed by cell death. An analogous strategy to overload glioblastoma cells with unrepaired DNA damage involves TMZ treatment with concurrent inhibition of MGMT in those cases where the MGMT gene promoter is not methylated.

An emerging alternative treatment strategy takes advantage of the synthetic lethality and the accumulated knowledge about the DDR mechanisms. This strategy exploits tumour-selective defects in certain DNA repair pathways, such as DSBR, by...
homologous recombination (HR). HR is a mechanism to copy a DNA sequence from an intact DNA molecule (mainly from the newly synthesised sister chromatid) to bypass or repair replication-associated DNA lesions. This promising strategy exploits HR defects that are found in some tumours. These HR-deficient tumours are particularly dependent on other repair processes to avoid the generation of DSBs. These tumour cells are therefore particularly sensitive to inhibition of these other repair processes. Such a strategy has shown promise in preclinical studies in which breast tumour cells defective to HR appear hypersensitive to inhibition of base excision repair by small molecule inhibitors of poly(ADP-ribose) polymerase (PARP). Of note, PARP inhibition has shown promise in glioblastoma treatment in cell culture models and several PARP inhibitors are under investigation in clinical glioblastoma trials.

**SUMMARY**

In this review, we have discussed key principles underlying the current development of glioblastoma therapeutics. Emphasis was placed on conceptual frameworks rather than specific drugs or targets. These principles should serve as the basis for translating fundamental biological tenets into clinically useful therapeutic strategies.

**Contributors**

CC and KN authored the sections on tumour heterogeneity (concept 1), oncogene addiction (concept 2), non-oncogene addiction (concept 3), non-coding RNAs (concept 6), CCC, BJ and BJ Jr authored the section on tumour initiating cells (concept 4) and DNA damage response (concept 7) and CCC, WF, BC and BJ Jr authored the section on tumour microenvironment (concept 5). The content of the final manuscript was reviewed by all the authors.

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Glioblastoma is the most common form of primary brain cancer and remains one of the most aggressive forms of human cancer. Current standard of care involves maximal surgical resection followed by concurrent therapy with radiation and the DNA alkylating agent, temozolomide. Despite this aggressive regimen, the median survival remains approximately 14 months. Meaningful strategies for therapeutic intervention are desperately needed. Development of such strategies will require an understanding of the framework of therapeutic concepts that have evolved over the past three decades. This article will review the key principles that drive the formulation of therapeutic strategies in glioblastoma. Specifically, the concepts of tumor heterogeneity, oncogene addiction, non-oncogene addiction, tumor initiating cells, tumor micro-environment, non-coding sequences, and DNA damage response will be reviewed.
INTRODUCTION

Glioblastoma is the most common form of primary brain tumor. The incidence of this tumor is fairly low, with 2-3 cases per 100,000 people in Europe and North America. It is one of the most aggressive forms of human cancer. Without treatment, the median survival is approximately 3 months. The current standard of treatment involves maximal surgical resection followed by concurrent radiation therapy and chemotherapy with the DNA alkylating agent, temozolomide. With this regimen, the median survival is approximately 14 months. For nearly all affected, the treatment remains palliative.

The best available evidence suggests that glioblastomas originate from cells that give rise to glial cells. These glial derived tumors are graded by the World Health Organization (WHO) into 4 categories, termed WHO grade 1 to grade 4. The higher grade denotes histologic features of increased malignancy. WHO 4 glioma is essentially synonymous with glioblastoma.

Studies carried out over the past three decades suggest that glioblastomas, like other cancers, arise secondary to the accumulation of genetic alterations. These alterations can take the form of epigenetic modifications, point mutations, translocations, amplifications or deletions and modify gene functions in ways that deregulate cellular signaling pathways leading to the cancer phenotype. The exact number and nature of genetic alterations and deregulated signaling pathways required for tumorigenesis remains an issue of debate, although it is now clear that CNS carcinogenesis requires multiple disruptions to the normal cellular circuitry. These genetic alterations result in either activation or inactivation of specific gene functions that contribute to the process of carcinogenesis. Genes, that when activated, contribute to the carcinogenesis are generally termed proto-oncogenes. The mutated
forms of these genes are referred to as oncogenes. Genes, that when inactivated, contribute to the carcinogenesis are termed tumor suppressor genes.

Recent research in the area of experimental and clinical oncology has identified the key signaling pathways, critical regulatory nodes, genes and their protein products, as well as their mutual cross-talks, thereby providing a solid molecular basis for selection of candidate therapeutic targets and drug discovery programs. These lines of investigation complement the recent efforts to sequence entire genomes of a growing number of human tumors including glioblastoma. The efforts have led to the formulation of new concepts and principles in tumor cell biology. Exploitation of these major advances have begun to provide exciting leads to conceptual framework that afford innovative therapeutic strategies. This article will aim to review these critical concepts and their relevance for glioblastoma therapeutic development.

**CONCEPT 1: GLIOBLASTOMA SUBTYPES**

There is an old adage that cancer is a hundred diseases masquerading in one. While this adage is based on clinical and pathologic observations, systemic genomic characterization of a large number of glioblastoma specimens confirms the notion that subtypes with distinct pathologic molecular events and therapeutic responses exist.

The Cancer Genome Atlas project (TCGA) is a major NIH initiative involving institutions spanning the continental U.S. with the goal of tumor specimen collection and molecular characterization. Glioblastoma was one of the first tumor types characterized in this effort. This vast wealth of data is unprecedented, and despite the enormous challenge to process and analyze this incoming information, correlations of such emerging ‘genetic and expression profiles’ or ‘tumor landscapes’ with tumor
biology and clinico-pathological features of the patients including therapeutic responses are beginning to impact oncology.

These studies have led to the understanding of glioblastoma as an umbrella term that encapsulates subtypes characterized by distinct molecular properties. Based on global transcript profiling, glioblastoma can be divided into three to four distinct subtypes. Interestingly, each subtype harbors distinct genetic aberrations and proteomic profiles. The recognition that glioblastoma consists of subtypes varying in molecular circuitry and biologic behavior suggests that no therapy can be universally efficacious. The major importance of this concept of heterogeneity is that meaningful therapeutic gain can only be attained by customizing the therapy to the underlying molecular circuit. One subtype (termed classical by the TCGA and proliferative by Philips et al.) is characterized by frequent amplification or mutations in the Epidermal Growth Factor Receptor (EGFR) gene. In contrast, in another subtype, termed proneural by both groups, harbors frequent mutations in p53, Platelet Derived Growth Factor Receptor A (PDGFRA), and Isocitrate Dehydrogenase 1 (IDH1). A third type, termed mesenchymal, is characterized by frequent mutations in the Neurofibromatosis type 1 gene (NF-1).

Importantly, these transcriptomal subtypes appear to differ in their clinical courses and therapeutic responses. In terms of prognosis, studies by Philips and Verhaak both demonstrated increased overall survival in patients afflicted with proneural glioblastoma relative to other molecular subtypes. In terms of therapeutic response, Verhaak et al. explored this issue by stratifying the patients with various molecular subtypes into two groups: 1) those that received concurrent chemo-radiation therapy or received >3 cycles of chemotherapy and 2) those that did not receive concurrent chemo-radiation therapy or received <4 cycles of
chemotherapy. When stratified this way, the authors found that the two groups exhibited comparable survival in the pro-neural group. In contrast, for other molecular subtypes, patients in group one exhibited improved survival relative to group 2. Since the analysis combined the survival effect of concurrent chemo-radiation therapy and prolonged chemotherapy, it is difficult to assess whether the effect is due to the former or the latter. Taken as a whole, these data sets suggest that the patients with the pro-neural glioblastomas tend to survive longer but are less responsive to conventional chemotherapy or chemo-radiation therapy.

CONCEPT 2: ONCOGENE ADDICTION

The term “oncogene addiction” was initially coined by Dr. Bernard Weinstein to describe the phenomenon that some tumors exhibit exquisite dependence on a single oncogenic protein (or pathway) for sustaining growth and proliferation. Such dependence has been convincingly demonstrated in both tissue culture and transgenic mice systems for oncogenic versions of MYC and RAS. Application of this concept to the clinical setting has achieved variable success in various cancer types, including chronic myelogenous leukemia (CML) harboring the BCR-ABL translocation, Erb2 over-expressing breast cancer, and Non-Small Cell Lung Cancer (NSCLC) harboring a subset of EGFR mutations. A simplistic application of this concept in glioblastoma would involve identification of the critical “addicted” oncogene followed by the inhibition of such oncogene(s). Unfortunately, the actual biology of glioblastoma is far more complex.

To understand this complexity, a careful analysis of the fundamental notion of oncogenic addiction is needed. In some ways, the observation that tumors exhibit
dependence on a particular oncogenic pathway at some point in its history is not surprising. However, considering the plethora of dynamic genetic changes that accumulated during cancer progression, it is somewhat counter-intuitive to suspect that any particular pathway would play a prominent role in maintaining cell viability. Moreover, inactivation of the normal counterpart of the addicted oncogenic protein is often tolerated in normal tissue. These observations suggest that the genetic circuitry of the cancer cell have been extensively re-programmed to result in this “addicted” state.

The molecular nature of this re-programming remains poorly understood. Several hypotheses have been put forward. One hypothesis involves the notion of “genetic streamlining”, where genetic instability in cancer cells is thought to mutationally or epigenetically inactivate certain signaling pathways that are operational in a normal cell but not required for growth in the cancer cell. In this “streamlined” state, the tumor cell becomes hyper-dependent on the oncogene driven processes. A more generalized form of this explanation involves the notion of synthetic lethality. Two genes are considered synthetically lethal if cells remain viable with inactivation of either gene. Simultaneous inactivation of both genes, on the other hand, results in cell death. It is thought that the cancer cells have accumulated mutations that are synthetically lethal with the absence of critical oncogenes. The main difference between this hypothesis and the “streamline” hypothesis is that the mutation in the former can result in a gain or loss of function, whereas the later specifically proposes a loss of function. A third hypothesis suggests that oncogenes reprogram the tumor cell by both pro-survival and pro-apoptotic signaling. With acute inactivation, the pro-survival signaling decayed faster than the pro-
apoptotic signaling, resulting in tumor death. This thesis has been coined the “oncogene shock” hypothesis.\textsuperscript{23, 25, 26}.

The main reason for revisiting the framework of oncogene addiction is to discuss the mechanism by which the cells can evolve to avoid such addiction. For instance, in the context of synthetic lethality, EGFR inhibition may be cytotoxic to glioblastoma cells only in the appropriate genetic context. Indeed, therapeutic effects of EGFR inhibition were observed only in patients with tumors expressing an oncogenic form of EGFR and an intact PTEN tumor suppressor gene.\textsuperscript{27} To complicate the matter, recent studies demonstrate that glioblastomas harbor activation of multiple oncogenic Receptor Tyrosine Kinases (RTKs), such that inactivation of any single oncogene merely diverts signaling through other active oncogenes.\textsuperscript{28} In these contexts, it is evident that meaningful therapy will require simultaneous inhibition of multiple oncogenes or identification of the fitting genetic context.

CONCEPT 3: NON-ONCOGENE ADDICTION

Emerging literature suggests an alternative strategy to the multi-target approach. These studies reveal that oncogene activation introduces secondary physiologic changes that stress cellular capacity for survival. Consequently, tumor cells becomes more dependent (or hyper-dependent) on processes required to compensate for these stressful conditions.\textsuperscript{29, 30} This phenomenon is termed “non-oncogene addiction” since the compensatory processes required for tumor survival do not directly contribute to the cancer formation. In other words, even the genes that are not themselves targeted by tumorigenic mutations may well become essential for the tumor to survive the stressful environment and fuel the demanding process of tumor
progression. Consequently, interfering with the function of such genes could cause tumor kill while sparing the normal counterpart. The principle of non-oncogene addiction suggests that there is a wider spectrum of therapeutic options than afforded under the paradigm of “oncogene addiction”. In many cases, compensatory processes involved in “non-oncogene addiction” are the same as those that basic scientists have studied for years (for instance, DNA repair). Mechanistic investigations into these biologic processes by the basic scientists have yielded a rich database of inhibitors. Thus, identifying gene functions that compensate for oncogene induced cellular stress should afford opportunities to tap into this rich database and expand the denominator of drugs

There are several examples of such critical non-oncogenic pro-survival functions required for the maintenance of the tumorigenic state in glioblastoma. EGFR is a critical proto-oncogene in glioblastoma pathogenesis. Our laboratory has demonstrated that EGFR hyperactivation results in an increased accumulation of reactive oxygen species (ROS), which in turn cause cytotoxic DNA damage. To compensate for the deleterious effect of ROS, EGFR hyperactive glioblastomas exhibit increased reliance on DNA repair process required for the repair ROS related DNA damage. Selective targeting of EGFR hyperactive glioblastomas can, thus, be achieved by inhibition of these repair process. Other groups have demonstrated that EGFR hyperactivation in glioblastoma cell lines heightens requirement for lipogenesis. Additional examples of such critical non-oncogenic pro-survival functions required for maintenance of the tumorigenic state include dependency on mechanism for compensating mitotic and proteotoxic stress and interplay with the tumor microenvironment including the immune system.
available for combinatorial therapy. Identifying genes that are synthetically lethal with oncogenes constitute an attractive means to this end.

It is important to note that effects of therapies designed based on the principles of “oncogene addiction” and of “non-oncogene addiction” are inherently antagonistic. For instance, EGFR inhibition leads to a reduction of ROS, obviating the need for DNA repair. In this context, the combination of DNA repair inhibition and EGFR inhibition would not be desirable. Rational strategies for synthesizing the two therapeutic paradigms remains a major intellectual challenge.

**CONCEPT 4: TUMOR INITIATING CELLS**

Another advance that may profoundly change our thinking about solid tumors including glioblastoma involves the concept of tumor initiating cells. The experimental observation is that within a total population of glioblastoma cells, there appears to be a small sub-population of cells that are highly tumorigenic (hence the term “tumor initiating cells” or “TICs”), with capacity for self-renewal. In some studies where severely immune-compromised are used as assay for melanoma xenograft formation, the proportion of TICs within a tumor has been reported as high as 27%. To the extent that glioblastoma tumor initiating cells share many common properties when compared to neural stem cells, it is proposed that the TICs originated from stem cells. While there are some data supporting this hypothesis, the universality of this hypothesis remain controversial.

Protein markers to prospectively identify and isolate these putative TICs have been reported, such as the transmembrane glycoprotein CD133 (prominin-1) in glioblastomas. However, the value of CD133 as a single marker of glioblastoma TICs remains controversial, partly because also CD133-negative glioblastoma cells
could give rise to tumors in an intracranial mouse xenograft model. These uncertainties motivate an ongoing search for additional candidate TIC markers. Candidate cell surface molecules suggested in this context include the adhesion glycoprotein L1CAM, surface carbohydrate antigen CD15 (SSEA-1), surface marker A2B5, and integrin α6. Currently, there are no generally accepted cell surface markers for defining TIC. The definition of TICs remains a functional one as defined by the ability of a tumor cell to sustain self-renewal and initiate glioblastoma formation in immuno-compromised xenograft models.

Arguably, the most important aspect of the concept of TICs is that this population appeared particularly resistant to conventional radiation and chemotherapy. In this context, TICs may be responsible for glioblastoma recurrence after conventional therapy. Given such properties, it is understandable that glioblastoma research has recently focused on identification and development of potential anti-TIC therapies. Two of these strategies, namely targeting the TICs as part of a vascular niche, and attempts to overcome their therapeutic resistance, will be discussed in the following sections on glioblastoma angiogenesis and the role of DNA damage response pathways, respectively. Here, we briefly consider strategies that are emerging as potentially fruitful approaches to treat glioblastoma through targeting TICs.

The first strategy reflects the efforts to identify suitable cell surface markers to reliably identify glioblastoma TICs – with the hope of conjugating the corresponding antibody to cytotoxic compounds as therapeutic agents. The second strategy is based on observations that some TICs, like neural stem cells, can be induced into a differentiated state whereby the self-renewal properties are lost. Among the suggested agents to induce such TIC differentiation, the bone morphogenetic proteins (BMPs)
appear promising\textsuperscript{45}. The third strategy involves modulating specific signaling pathways required for maintaining the TIC state. Pathways targeted include those mediated by EGFR, Wnt-beta catenin, STAT3, Sonic Hedgehog-Gli, and Notch pathways\textsuperscript{46}. Finally, normal neural stem cells have been shown to migrate toward and track TICs. Based on this principle, neural stem cells have been used as delivery vehicles to increase local concentration of therapeutic agents in the vicinity of TICs\textsuperscript{47}.

**CONCEPT 5: TUMOR MICROENVIRONMENT**

Over the past two decades, conceptualization of glioblastomas has evolved from a collection of relatively homogenous cells to the recognition of distinct sub-populations of tumor cells to that of a complex organ, with constant interactions between tumor cells and aberrant stromal elements. Analogous to the distinct functions of different tissues in an organ, genomic characterization using cells derived from distinct regions of the tumor revealed genetic heterogeneity\textsuperscript{48}. A major concept in oncology has emerged that reciprocal signaling between the distinct subpopulations of neoplastic cells and the aberrant stromal elements serve to sustain progressive neoplastic transformation and possibly functional specialization\textsuperscript{49}. Understanding of these interactions has afforded novel therapeutic targets. For the purpose of this review, distinct subpopulation of neoplastic cells (tumor heterogeneity) and aberrant stromal interactions will both be considered as components of the microenvironment.

Studies of EGFR revealed a beautiful illustration of signaling between subpopulation of genetically distinct neoplastic cells in glioblastoma. EGFR\textsubscript{vIII} is a variant of EGFR that arose from spontaneous deletion of exons 2-7\textsuperscript{50}. This variant is present in about 20% of glioblastomas\textsuperscript{51} and results in constitutive hyper-activation
of EGFR. Clinical studies suggest that glioblastoma patients harboring this variant tend to exhibit worse prognosis. Interestingly, the vIII variant is rarely found in the absence of EGFR over-expression. Further, when found, the variant is typically present in only a subset of the total tumor mass. Investigations into the molecular mechanism underlying these observations revealed that EGFRvIII over-expression increased the secretion of interleukin 6 (IL6) and Leukemia Inhibitory Factor (LIF), two soluble cytokines. These cytokines trigger phosphorylation of gp130 in the non-EGFRvIII expressing cells, which in turn activate EGFR of these cells. This activation increases the tumorigenicity and aggressiveness of the cancer. Such signaling may serve to actively maintain tumor cell heterogeneity.

In addition to the signaling between distinct and genetically defined subpopulations of tumor cells, normal cells without genetic alterations associated with carcinogenesis are often recruited to the foci of tumor cells. In the process, these normal cells undergo phenotypic changes in response to direct physical interaction with cell surface proteins on the tumor cells or through interaction with secreted soluble factors. These changes result in the release of growth factors that further enable and sustain neoplastic transformation or lead to new blood vessel formation. Cycles of such reciprocal interaction facilitate stepwise progression in neoplastic progression.

In terms of the non-neoplastic cell types shown to facilitate neoplastic information, they can generally be divided into three categories. The first category involves endothelial cells or endothelial cell precursors. These cells are critical for tumor growth since there are inherent limitations on the distance that oxygen and macromolecules can travel. In xenograft models, solid tumors can only proliferate up to a size of 1-2 mm without the development of a new blood supply. Quiescent
endothelial cells in proximity of the neoplastic foci may be induced to initiate biologic programs that lead to blood vessel formation by secreted factors such as Vascular Endothelial Growth Factor (VEGR, see below)\textsuperscript{60}. Alternatively, endothelial cell precursors in the blood stream may be recruited into the tumor foci \textsuperscript{58, 61}. A final mechanism involves the trans-differentiation of TICs to become endothelial cells \textsuperscript{62}.

The second class of non-neoplastic cells that actively participate in tumor progression is fibroblasts. There is good evidence that these otherwise genetically normal fibroblasts, when in proximity of tumor cells, can become “re-programmed” to promote/sustain neoplastic transformation. Transplantation experiments mixing cancer-associated fibroblast with cancer cells lead to a more aggressive tumor phenotype than “normal” tumor cells. This tumor promoting activity is largely thought to be the combined effect of cell-to-cell interaction as well as cytokine release \textsuperscript{57}. Non-neoplastic astrocytes perform many of the functions associated with fibroblasts. Thus, the interactions between glioblastoma cells and non-neoplastic astrocytes warrant further investigations.

The final class of non-neoplastic cells recruited are cells that mediate immune function. In general, these cells may possess tumor antagonizing activity or tumor promoting activity \textsuperscript{49}. These divergent properties may be rationalized by understanding that the immune system is required for both the destruction of foreign cells as well as facilitating wound healing. Properties associated with the former will likely lead to tumor ablation \textsuperscript{63}. On the other hand, cytokines and growth factors associated with wound healing may promote tumor growth \textsuperscript{64}.

The glioblastoma cells have evolved a large number of mechanisms that allow escape from immune detection and ablation, including release of immunosuppressive
cytokines, such as interleukin (IL)-10, CTLA-4, and transforming growth factor-ß (TGF-ß) or expression of cell surface molecules that facilitate immunosuppression, such as B7-H1. These events, in turn, lead to the induction of regulatory T cells (Treg), down modulation of antigen-presenting cell, with concomitant loss of T-cell effector function; or loss of functional MHC class I receptors.

These factors contribute to a “hostile” microenvironment that compromises the immune cells ability to achieve tumor eradication. For instance, primed CD8\(^+\) cytotoxic T cells can penetrate the blood-brain barrier and access the central nervous system. However, they are incapable of tumor eradication. Indeed, in patients with glioblastoma, tumor progression is seen despite the presence of tumor-infiltrating lymphocytes (TIL).

Interestingly, glioblastoma’s capacity to suppress immune response appears intimately associated with the process of neoplastic transformation. Phosphatase and TENsin homologue (PTEN) encodes a tumor suppressing phosphatase that is frequently mutated during glioblastoma pathogenesis. The translation of many immune-suppressive cytokines and molecules, including IL10 and B7-H1, are under the regulation of PTEN. Thus, PTEN loss during neoplastic transformation leads to increased expression of immune-suppressive cytokines and cell surface molecules. This expression, in turn, creates a hostile environment for immune cells that otherwise target tumor for ablation.

Understanding the interaction between the genetically distinct subpopulation of glioblastoma cells and their microenvironment has yielded novel therapeutic developments. The endeavor most frequently cited in this regard involves
angiogenesis inhibitors. Realizing that VEGF is critical in angiogenesis, bevacizumab, a humanized antibody against VEGF, was developed. While there has not been a randomized control trial to assess the efficacy of bevacizumab, phase II clinical trial demonstrated improved progression free survival in recurrent glioblastomas (after concurrent temozolomide/radiation treatment) relative to historical data of patients who received temozolomide at recurrence. However, no overall survival benefit has been demonstrated with bevacizumab treatment. Clearly, angiogenesis inhibition is but one of the many strategies can be developed based on the concept of tumor microenvironment.

CONCEPT 6: NON-CODING DNA SEQUENCES

Classically, coding sequences are defined as the strand of DNA that has the same base sequence as the RNA transcript produced (with the caveat that thymines are replaced by uracil). While the identification of nucleotide alterations within the coding sequences of proto-oncogene or tumor suppressor genes has significantly contributed to our understanding of carcinogenesis, there is an emerging appreciation that alterations in non-coding sequences similarly contribute to carcinogenesis. A notable example involves the regulation of gene transcription by reversible modification of gene promoter regions – a phenomenon sometimes referred to as “epigenetic regulation.” Similarly, we are beginning to appreciate the importance of transcripts that do not encode for proteins but are transcribed, such as microRNAs and Long non-coding RNAs (or LincRNAs), in terms of both transcriptional and post-transcriptional modifications. The concept that non-coding DNA sequences regulate gene function and impact carcinogenesis has significantly
expanded the repertoire of strategies available for glioblastoma therapeutics. To review this concept, we will discuss illustrative examples of epigenetic regulation, microRNAs, and LincRNAs.

The term “epigenetic regulation” has been coined to describe the phenomenon that heritable changes in gene expression can occur in the absence of changes in the DNA sequences encoding for gene function \(^{77}\). The mechanism underlying this regulation involves cytosine methylation \(^{81}\) or histone modifications that, in turn, modulate the accessibility of gene promoter regions to transcriptional factors \(^{82}\). Cytosine methylation typically occurs in the context of CpG di-nucleotide repeats, or CpG islands \(^{81}\). Promoters harboring heavily methylated CpG islands are typically transcriptionally silenced. There are two types of promoter methylation that are particularly pertinent to glioblastoma therapy: methylation in the promoter region of the DNA repair gene, Methyl-Guanine Methyl Transferase (MGMT) and the glioma-CpG Island Methylator (G-CIMP) phenotype.

MGMT encodes an enzyme that removes alkyl adducts at the O6-position of guanine \(^{83}\). Because alkyl modification at this position is highly toxic and constitute the primary mechanism for the tumoricidal activity of the chemotherapeutic agent, temozolomide (TMZ), MGMT expression level correlates well with TMZ response in glioblastoma patients \(^{84}\). The human MGMT gene possesses a CpG island that spans approximately 1,000 bases around the transcriptional start site. Detailed analysis of this region revealed 108 CpG sites \(^{85}\) that are methylated. Methylation of a subset of these CpGs has been associated with transcriptional silencing of MGMT \(^{86,87}\) and is associated with improved clinical outcome in glioblastoma patients receiving TMZ therapy. Interestingly, MGMT promoter methylation is also associated with improved survival in patients who did not receive TMZ therapy \(^{88,89}\). While the mechanism
underlying this observation remains unclear, it seems likely that MGMT may participate in detoxifying the accumulation of endogenous DNA damage that is typically associated with the oncogenic state. As discussed in concept 7, glioblastoma cells accumulate endogenous DNA damage in the absence of DNA damaging agents. These endogenous DNA damages are not unlike those induced by temozolomide or radiation in that they could trigger cell death if unrepaired. Thus, tumors with high levels of MGMT may grow more robustly since MGMT is capable of detoxifying these endogenous DNA damages. If the tumor cells grow more robustly, the patient will survive for a shorter duration. In contrast, the glioblastoma cells with low MGMT may be more susceptible to the deleterious effects of the endogenous DNA damages. These tumors may grow less robustly, resulting in longer patient survival.

The G-CIMP phenotype refers to the observation that a subset of glioblastomas exhibits concerted CpG island methylation at a large number of loci. Since genes required for tumor growth are located at many of these loci, glioblastomas harboring the G-CIMP phenotype tend to be more benign. Correspondingly, patients with G-CIMP glioblastomas experienced significantly improved outcome. Understanding the concept that the patterns of CpG island methylation directly impact outcomes in glioblastoma patients open the door to therapeutic strategies aimed at enhancing promoter methylation at select promoter loci. Importantly, recent studies suggest that promoter methylation at distinct loci may be affected by specific chromatin modulating factors.

MicroRNAs (miRNAs) are small non-coding RNAs of 20-22 nucleotides that, through imperfect pairing, bind to the 3’ untranslated regions (UTR) of protein-
coding mRNAs. Typically, this binding leads to mRNA degradation or inhibition of protein translation to suppress the expression of the target proteins \(^7^8\). Bioinformatic analysis predicts that a single miRNA can potentially regulate hundreds of target onco- or tumor suppressor proteins. Expectedly, miRNAs have been implicated in carcinogenesis and resistance to chemotherapy \(^7^8\). As one illustrative example, our laboratory recently demonstrated that the protein MGMT is under the regulation of miR-181d \(^9^2\). Cell biologic studies revealed that binding of miR-181d to the 3'UTR of MGMT caused decreased MGMT expression. This inverse relationship was validated in glioblastoma specimens. Importantly, patients with high miR-181d expression (hence low MGMT) are more likely to respond to TMZ chemotherapy.

LincRNAs are transcripts > 5 kb that are evolutionarily conserved across mammalian genomes. These RNAs are transcribed by Polymerase II but do not encode proteins. The LincRNAs serve to suppress transcription by targeting chromatin-modifying complexes to specific genomic loci \(^7^9,^8^0\). While the role of LincRNA in glioblastoma awaits careful scrutiny, LincRNA have been shown to mediate the function of tumor suppressor genes pertinent to glioblastoma pathogenesis. As one example, \(TP53\) encodes a transcription factor that regulates gene sets critical for cell cycle progression and apoptosis. Under normal conditions, p53 is a short-lived protein \(^9^3\). In response to cellular stress (for instance, DNA damage or oncogene expression), p53 undergoes post-translational modifications and protein-protein interactions that enhance its stability and transcriptional activity \(^9^3\). One of the down-stream effectors of p53 is a LincRNA. This LincRNA serves as a key mediator to suppress transcription of other p53 effectors \(^9^4\). Such mechanisms may be operational in glioblastomas.
Understanding the concept that non-coding sequences play critical roles in glioblastoma pathogenesis and resistance to chemotherapy offer novel strategies for biomarker development and therapy. For instance, direct introduction of select miRNAs into glioblastoma has been shown to inhibit growth and proliferation\textsuperscript{95}. Similarly, incorporation of miR-181d expression level may further augment the predictive value of MGMT promoter methylation. Importantly, the concept predicts certain situations where the effects of an oncogenic mutation can be voided by the effects of non-coding sequences. Integrating the biology of non-coding sequences in the context of mutational profile will be critical in understanding tumor physiology and meaningful therapeutic development.

**CONCEPT 7: DNA DAMAGE RESPONSE**

From a broader perspective, the status of the molecular machinery that detects, signals and repairs DNA damage, and overall orchestrates the multifaceted cellular response to genotoxic insults (here referred to as the DNA damage response: ‘DDR’\textsuperscript{96}) critically impacts both tumor development and clinical outcome. While this is arguably relevant for any type of tumor to some extent, the DDR concept is particularly important for glioblastomas for the following reasons. First, the standard-of-care nonsurgical modalities used to treat glioblastomas, namely ionizing radiation and TMZ-based chemotherapy, operate through their genotoxic effects by causing mainly DNA double strand breaks (DSB) and alkylated DNA lesions, respectively. Therefore, each patient’s germ-line disposition of the DDR-related genes, along with any somatic alterations within the DDR machinery that have been selectively acquired by the tumor dictate (along with other factors such as the tumor microenvironment
discussed above) the response of individual glioblastoma patients to such therapies. Second, among the hallmarks of glioblastomas is their resistance to radiotherapy and chemotherapy. These phenomena highlight the intimate involvement of the cellular DDR network, particularly DNA damage signaling, cell-cycle checkpoints and DNA repair pathways, in the pathobiology of glioblastomas. Third, the harmful side-effects of the standard therapies, including brain damage and consequently cognitive changes, are also attributable to DNA damage and the cellular and tissue responses to such treatments. Fourth, genetic and/or epigenetic aberrations of a range of DDR factors, including the above mentioned p53 tumor suppressor or DNA repair genes such as MGMT, occur commonly during glioblastoma pathogenesis and/or upon treatment. This aspect of gliomagenesis has been suspected and partly known for years, however, it has only been validated by the recent insights gained through comprehensive analyses by complete tumor genome sequencing within the framework of the TCGA initiative. Finally, the TICs (see Concept 4), appear to be particularly resistant to DNA-damaging therapies. This resistance is, at least in part, due to enhanced DNA damage signaling and checkpoint machinery.

Conceptually very relevant for such DDR-related features of gliomas is the recently described strong, constitutive activation of the DDR signaling pathways, observed from the early stages (grade II gliomas) of gliomagenesis up to glioblastomas. This spontaneous DDR activation precedes any genotoxic treatment, and it appears to be even more pronounced in gliomagenesis than in early lesions of major epithelial tumor types, where this phenomenon represents a candidate intrinsic barrier against activated oncogenes and tumor progression. A major source of such DDR activation in early lesions including low-grade gliomas appears to be oncogene-induced replication stress, while in later stages
of tumor progression, particularly in glioblastomas, the constitutive DNA damage signaling is fueled by both continued replication stress and also by enhanced oxidative stress. Biologically, such oncogene-evoked DDR activation often leads to cell death or permanent proliferation arrest known as cellular senescence. This activation eliminates nascent tumor cells from the proliferative pool, thereby delaying or preventing tumor progression. Those lesions that do progress in the face of such constitutively activated DDR often do so due to selection of various defects along the DDR signaling or effector pathways, such as mutations in the ATM-Chk2-p53 DDR pathway. Importantly, while such selected DDR aberrations facilitate tumor progression by allowing escape from DDR-induced senescence or apoptosis, the very same defects may create tumor-specific vulnerabilities that can be exploited by therapeutic strategies based on the synthetic lethality principle.

In terms of exploiting the status of the DDR machinery for glioblastoma therapies, two major avenues are under intensive research and validation. First, there are promising attempts to sensitize glioblastoma cells (including the more resistant TICs) to conventional genotoxic therapy, such as ionizing radiation, by concomitantly inhibiting the DNA damage signaling to downstream checkpoint and repair effectors. This strategy relies mainly on small molecule inhibitors of DDR kinases ATM; ATR, Chk1 and Chk2. This strategy appears particularly suitable for tumors with mutant p53. Such cancer cells lack the major p53-dependent G1/S checkpoint, and upon inhibition of the DDR kinases (whose activity underlies the still operational G2/M checkpoint) enter mitosis with an overload of unrepaired DNA damage, both endogenous and therapy-induced,
followed by cell death. An analogous strategy to overload glioblastoma cells with unrepaired DNA damage involves temozolomide treatment with concurrent inhibition of MGMT in those cases where the MGMT gene promoter is not methylated.

An emerging alternative treatment strategy that takes advantage of the synthetic lethality and the accumulated knowledge about the DDR mechanisms. This strategy exploits tumor-selective defects in certain DNA repair pathways, such as the DSB repair by homologous recombination (HR). HR is a mechanism to copy a DNA sequence from an intact DNA molecule (mainly from the newly synthesized sister chromatid) in order to bypass or repair replication-associated DNA lesions. The promising strategy exploiting HR defects that are found in some tumors. These HR deficient tumors are particularly dependent on other repair processes to avoid the generation of DSBs. These tumor cells are, thus, particularly sensitive to inhibition of these other repair processes. Such strategy has shown promise in preclinical studies where breast tumor cells defective to HR appear hypersensitive to inhibition of base excision repair by a small molecule inhibitors of poly(ADP-ribose) polymerase (PARP). Of note, PARP inhibition has shown promise in glioblastoma treatment in cell culture models, and several PARP inhibitors are under investigation in clinical glioblastoma trials.

**SUMMARY**

In this review, we have discussed key principles underlying current development of glioblastoma therapeutics. Emphasis was placed on conceptual
framework rather than specific drugs or targets. These frameworks should serve as the basis for translating fundamental biologic tenets into clinically useful therapeutic strategies.

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REFERENCES


REVIEWER 1

You have done a nice job reviewing important concepts. My principal concern is that you presuppose a firm understanding of cancer biology that may elude most of the readership. I would like to see you explain the basic biologic underpinnings more clearly. For example, for concept 7, many readers may not be familiar with DSBs, PARP inhibitors, homologous recombination, etc.

Response: We appreciate this comment and have modified the text of Concept 7 accordingly. In the revised manuscript, we not only explain the three terms highlighted by the referee (DSB, PARP inhibitors, homologous recombination), but also additional ones that might be less familiar to the readers. At the same time, such explanations cannot be too detailed, given the restrictions on valuable journal space (referee no. 3 recommended an overall shortening of the manuscript), and the fact that references are indicated that can help readers to find more information, if desired. We are also pleased to note that reviewer 3 particularly liked the description of Concept 7.

Some minor comments:
Page 4, line 56 - this sentence does not make sense.

Response: We thank the reviewer for this comment. The sentence “While this adage is based on clinical and pathologic observations, systemic genomic characterization of a large number of glioblastoma specimens (The Cancer Genome Atlas project: TCGA) confirms the notion that subtypes with distinct pathologic molecular events and therapeutic response“ has been modified to “While this adage is based on clinical and pathologic observations, systemic genomic characterization of a large number of glioblastoma specimens (The Cancer Genome Atlas project: TCGA) confirms the notion that subtypes with distinct pathologic molecular events and therapeutic response exist.“ This change can now be found on page 4 of text.

Page 6, line 3 (and accompanying figure) - I don't think the Verhaak paper really demonstrates that patients with the proneural subtype don't benefit from RT/TMZ. All it shows is that they don't benefit from prolonged post-RT chemotherapy.

Response: We thank the reviewer for raising this important point. The Verhaak dataset segregated the patients with various molecular subtypes into two groups: 1) those that received concurrent chemo-radiation therapy or received >3 cycles of chemotherapy and 2) those that did not receive concurrent chemo-radiation therapy or received <4 cycles of chemotherapy. When stratified this way, the authors found that the two groups exhibited comparable survival in the pro-neural group. In contrast, for other molecular subtypes, patients in group 1 exhibited improved survival relative to group 2. (Figure 5, Reference 13). Since the survival effect of concurrent chemo-radiation therapy and prolonged chemotherapy was grouped in the Verhaak analysis, it is difficult to assess whether the effect was due to the former or the latter. In this context, we have modified the text in our review to reflect the data rather than attribute the survival effect to either concurrent chemo-radiation or prolonged chemotherapy. This change can now be found on page 16 of text.
Page 15, line 50: this is inaccurate. First, these are phase II clinical trials of bev, not case series. Secondly, the studies were not designed to assess overall survival. Finally, the improved PFS was really compared to historical controls with ineffective chemotherapy (which included TMZ, which never got approval for recurrent GBM).

Response: We thank the reviewer for raising this point. The thesis that we wished to present is that bevacizumab has not been subjected to the rigor of randomized control trial. We have corrected the information to emphasize that the bevacizumab clinical data were grounded on phase II clinical trials, comparing progression free survival to historical controls of patients who received temozolomide at recurrence. This change can now be found on page 16 of text.

REVIEWER 2

The manuscript is a review of glioblastoma pathobiology. It is well written and contains the key elements. As a review, it is not particularly novel, but provides a compelling point of view.

Response: We appreciate the reviewer’s kind remarks.

REVIEWER 3

The authors aim at providing a scholar review on concepts and hallmarks of cancinogenesis, and potential strategies for treatment of glioblastoma. Although the topic and outline of the paper is timely and attractive, they fail to a large extent to meet their goals and respond to the promise of the title. The text is to a large extent lengthy and in parts “boring”, inhomogenous (e.g. concept 7 is a favorable exception), often sensational using buzz words without providing the scientific insight. The illustrations are not really helpful, while an original art depicting their “concepts and targets” is missing. The next would greatly gain by shortening and English language revision. Repeately, sentence are incomplete or meaningless.

Response: We apologize for the lengthy discussions and the meaningless sentences. We have gone through the text to shorten the text where appropriate and refine the language of the manuscript.

Specific Comments:
I would propose to change the title to New concepts in glioblastoma therapy. I don’t see why stating the 7 is informative or important. (It is like if Hanahan et al would be saying that there are x hallmarks of cancer.)

Response: In accordance to the reviewer’s recommendation, we have modified the title of review to “key concepts in glioblastoma therapy”
There are cases where the phrases lack any meaning (for example: page 4 line 56-60: …, systemic genomic characterization of a large number of glioblastoma specimens confirms the notion that subtypes with distinct pathologic molecular events and therapeutic response. This is meaningless. There are also repetitions of sections (Page 4 line 8-13 Despite some progress…) It has already been stated.

Response: We thank the reviewer for these comments. The sentence “While this adage is based on clinical and pathologic observations, systemic genomic characterization of a large number of glioblastoma specimens (The Cancer Genome Atlas project: TCGA) confirms the notion that subtypes with distinct pathologic molecular events and therapeutic response“ has been modified to “While this adage is based on clinical and pathologic observations, systemic genomic characterization of a large number of glioblastoma specimens (The Cancer Genome Atlas project: TCGA) confirms the notion that subtypes with distinct pathologic molecular events and therapeutic response exist.“ This change can now be found on page 4 of text.

In accordance to the reviewer’s comment, the redundant information on page 4 lines 8-13 has been deleted to achieve a shortened manuscript.

Page 3 line 58 genes when inactivated or activated and contribute to carcinogenesis are always and not generally called oncogenes and tumor suppressors.

Response: We have incorporated the reviewer’s comment into our text.

Concept 1 Phrase without sense: This profiling approach… Consider adding to concept 1: that while subtypes are predictive they not seem to be prognostic.

Response: We have revised the phrase in question to the following “These studies have led to the understanding of glioblastoma as an umbrella term that encapsulates subtypes characterized by distinct molecular properties.”

The aggregate of the data suggests that the transcriptome based molecular subtypes are both predictive and prognostic. Philips et. al. (Reference 12) as well as Verhaak et. al. (Reference 13) both yielded evidence that patients with the pro-neural subtype of glioblastoma survive longer than those with other molecular subtypes. Verhaak et. al. (Reference 13) further demonstrated that the patients with pro-neural subtype of glioblastoma tend not to benefit from concurrent chemo-radiation therapy or prolonged chemotherapy. We have further clarified this on page 5.

Concept 2. When is a cell hyper-dependent and when only dependent? Please avoid hyper-dependence. Hyper-activation though does exist.

Response: We thank the reviewer for this critical question. We define “hyper-dependence” as a term to describe a situation where the tumor cell is more dependent on a particular process than the non-neoplastic cell. The definition can now be found on page 9 of the manuscript.
Please provide the proper references to genetic streamlining etc. and not only the review of Sharma et al from Genes Dev. 2007 Dec 15;21(24):3214-31. Also if you name the first two theories why not give the name of the third namely, oncogenic shock (Sharma and Settleman 2006)?

**Response:** We have cited two other 2006 papers by the Settleman group discussing the hypothesis of “oncogenic shock”. We also included “oncogenic shock” as a terminology in the manuscript per the reviewer’s request (page 8).

Page 6 line 51. Not anti-intuitive but contra-intuitive

**Response:** According to Merriam-Webster’s Collegiate Dictionary, the proper term is neither anti-intuitive nor contra-intuitive. The correct term of “counter-intuitive” has been changed on page 7.

Concept 3. No comment, correctly written

**Response:** We thank the reviewer for the kind review

Concept 4. There is clear evidence also against TICs which should be stated including Quintana et al Nature. 2008 Dec 4;456(7222):593-8. Also Indar Verma’s group has shown that it is also true for glioblastoma. It is exactly the current test (glioblastoma formation in xenografts) which is limiting the understanding of the true nature of TICs.

**Response:** We thank the reviewer for this comment. We whole-heartedly agree that the current technology is limited for the study of TICs. However, with all due respect, we disagree with the reviewer on the interpretation of the Quintana paper as clear evidence against the existence of TIC. The paper reports that, on average, 27% of single cell suspensions derived from melanoma patients are capable of forming xenograft tumors when implanted into severely immunocompromised mice. The data may suggest that the prevalence of TICs in melanoma cells may be higher than previously thought when severely immunocompromised mice are used as an assay for assessing TIC activity. However, this data set does not constitute evidence against the existence of TICs. This discussion has been appended on page 10.

To our best knowledge, Dr. Verma’s result has not been published in a peer-reviewed journal. In this context, we cannot include this information in the current review.

Concept 5. In concept 5 the authors mix up tumor heterogeneity with microenvironment. Both are important but in the current presentation it is misleading. It is not clear from the text which cells express IL6 and LIF and instead of transactivation I would suggest to use paracrine activation as it is noted correctly later. The authors explain endothelial cells but there is no sign of the VEGF signaling and when actually they present VEGF there is no explanation what it could be.
Response: We thank the reviewer for this comment. We had intentionally conceptualized heterogeneity in tumor cell as part of the tumor microenvironment. This thesis has been better stated on page 12.

We apologize for not clearly stating the source of IL6 and LIF. These factors are secreted by EGFRvIII cells. This information has been appended on page 13.

The discussion of VEGF signaling was moved up to page 14 in accordance to the reviewer’s comment.

PTEN modulates Akt phosphorylation and not the S6Kinase. The effect of S6 kinase is indirect as it is lower on the pathway. Many other signaling regulates S6 kinase. As written PTEN is regulating the immune inhibitory cytokines but it is not clear which way therefore it is not at all necessary (as written on page 15 that PTEN loss will increase IL10 and B7-H1).

Response: We thank the reviewer for the suggestion of revision and have revised accordingly on page 15.

The authors suggest that there were case series comparing temozolomide with bevacizumab, but none of the provided references appear correct. Indeed the references presented are about bevacizumab +/- irinotecan in recurrent glioblastoma, after failure of temozolomide. Therefore the two cannot be compared.

Response: We thank the reviewer for bringing up this important point. The information has been corrected to “While there has not been a randomized control trial to assess the efficacy of bevacizumab, phase II clinical trial demonstrated improved progression free survival in recurrent glioblastomas (after concurrent temozolomide/radiation treatment) relative to historical data reported based on patients who received temozolomide at recurrence”. This can be found on page 16 of the revised manuscript.

Concept 6. In concept 6 authors mix up again two distinct phenomena, non-coding RNA with epigenetic modifications. These should be discussed as separate entities. From the later the authors arbitrary choose miRNAs and lincRNAs. It should be specified that this are a part of 2 bigger groups of non-coding RNA. LincRNAs (large intergenic non-coding RNA which should be noted in the text) are a part of the family of LncRNAs, long non-coding RNAs, whereas miRNAs is a part of the short regulatory RNAs including, siRNAs, piRNAs and snoRNAs. All this are potentially important in carcinogenesis.

Response: We appreciate this comment. For our review, we adopted the classical definition of coding sequence as the strand of DNA that has the same base sequence as the RNA transcript produced (with the caveat that thymines are replaced by uracil). To the extent that promoter regions are not part of this coding sequence, we discussed promoter methylation under the general heading of non-coding sequences. This discussion has been appended on page 16 of the revised manuscript.
We recognize the distinction between LincRNAs and miRNAs. To the extent that these sequences are non-coding by the classic definition, we discussed these entities in the section of non-coding RNAs.

**Page 17 line 3** There are no different patterns of promoter methylation. MGMT methylation is a single marker of TMZ responsiveness and it is not a pattern, while G-CIMP is a phenotype presented by a subgroup of patients.

**Response:** We appreciate the reviewer’s comment that MGMT promoter methylation and G-CIMP phenotype are distinct biomarkers for glioblastoma patients. We had conceptualized these events as distinct patterns of CpG island methylation. To accommodate the reviewer’s perspective, we have modified the sentence to “There are two types of promoter methylation that are particularly pertinent to glioblastoma therapy” on page 17 of the revised text.

**Page 17 line 38. The sentence is non-sense and does not explain the better prognosis of MGMT methylated patients.**

**Response:** We apologize for not explaining this concept more clearly. As explained in concept 7 and seen in reference 30, glioblastoma cells accumulate endogenous DNA damage in the absence of DNA damaging agents. These endogenous DNA damages are not unlike those induce by temozolomide or radiation in that they could trigger cell death if unrepaired. Thus, tumors with high levels of MGMT may grow more robustly since MGMT is capable of detoxifying many of these endogenous DNA damages. If the tumor cells grow more robustly, the patient will survive for a shorter duration. In contrast, the glioblastoma cells with low MGMT may be more susceptible to the deleterious effects of the endogenous DNA damages. These tumors may grow less robustly, resulting in longer patient survival. This explanation has been incorporated into page 18 of the revised text.

**Page 18 How would you selectively enhance promoter methylation? Please explain. LincRNA,: please specify what it stands for. Change: These RNAs do not encode for proteins… to these RNAs are transcribed by Pol II but do not encode proteins. If LincRNA is not yet implicated in GMB why discuss?**

**Response:** We thank the reviewer for this question. Recent studies suggest that promoter methylation at distinct loci may be affected by specific chromatin modulating factors. We have included this into the discussion on page 18.

LincRNA has been defined as Long Non-Coding RNA.

The suggested change (These RNAs do not encode for proteins… to these RNAs are transcribed by Pol II but do not encode proteins.) has been made on page 19 of the revised text.

To the extent that LincRNA play important roles in mediating p53 functions, and p53 plays a pivotal role in the pathogenesis of glioblastomas, we feel that a discussion of LincRNA is warranted. This discussion is added to page 19 of the revised text.
Concept 7 well written, no comment

**Response:** We thank the reviewer for the kind review