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CHAPTER I

INTRODUCTION TO CANCER AND GLIOMA

1. CANCER

In 2008 (most recent data) 0.5% of the 304,059,724 people in the United States developed an invasive form of cancer. Breast cancer (inc. 184,450 , † 40,930), colon and rectum cancer (inc. 148,810, † 49,960), lung cancer (inc. 215,000, † 161,840), and prostate cancer (inc.186,320, †28,660) appear to be the most common forms. Of the 1,437,180 people who developed cancer, around 21,810 got brain and/or other central nervous system tumors with a corresponding death rate of 13,070; which is more than half of the incidence in that year. When studying these numbers, one can see that apart from central nervous tumors, lung cancer too has a relatively high death rate. However, one should take into consideration that lung cancer often occurs at a higher age than brain cancer cases.[1]

In 2008 in The Netherlands an incidence of 90,182 (0.5%) of invasive cancer cases was reported on a population of 16,446,000 people in total. The most common type of cancer was breast cancer, with an incidence of 13,121 and a death rate of 3357. Similar to the US the other most common types of cancers were lung cancer (11,507, † 10,339), prostate cancer (10,512, † 2,476), and colon cancer (19,654, † 12,202). Cancer of the central nervous system had an incidence of 1156 and a death rate of 992.[2] As one can see, the numbers in The Netherlands reflect those of the United States.[2,3,1] The conclusion that can be drawn from these numbers is that in order to prevent death extensive research is needed for early diagnosis and treatment of invasive cancer in general. A particular high focus is necessary for the deadly central nervous system tumors.

Table 1.: Cancer incidence and death rates for the USA and The Netherlands (2008).

USA	Incidence	Death	% Death	*The Netherlands*	Incidence	Death	% Death
Breast	184,450	40,930	22.19030	**Breast**	13,121	3,357	25.58494
Colon	148,810	49,960	33.57301	**Colon**	19,654	12,202	62.08405
Lung	215,000	161,840	75.27442	**Lung**	11,507	10,339	89.84966
Prostate	186,320	28,660	15.38214	**Prostate**	10,512	2,476	23.55403
CNS	21,810	13,070	59.92664	**CNS**	1,156	992	85.81315

Abbreviations: USA, United States of America; CNS, Central Nervous System.

Cancer is simply stated by the transformation of a healthy cell into a malignant cancer cell. This malignant transformation can be predisposed by genetic factors, but

environmental influences mostly underlie the process of cancer. When environmental factors are the cause, think of certain chemicals, radiation or biological causes such as bacteria and viruses. It is important to take a closer look at the development of cancer cells, namely, which genes are involved? We can separate at least four groups of genes involved in this malignant transformation: oncogenes, tumor suppressor genes, DNA repair genes, and the gene encoding telomerase. The first oncogenes were discovered ironically with the help of viruses. It was rationalized that if a virus could be held responsible for the onset of cancer, then the genome of that virus should contain the responsible sequences for that onset. Furthermore, in the 70's Bishop and Varmus discovered that normal human cells too could have these similar gene sequences without the process of any malignancy. Nevertheless, these genes were as a matter of fact involved in the regulation of cell growth and differentiation and could therefore be defined as pro-oncogenes. Sometimes, numerous copies of those oncogenes occur in a cancer cell such as the c-erb-B2 gene which is repeated in a certain type of breast cancer or N-myc which can have an important role in the development of the neuroblastoma. Other processes associated with these oncogenes are chromosomal translocation, point mutations and viral infection, with the latter due to the incorporation of a virus into the human genome.

Tumor suppressor genes can be held responsible for the onset of a cancer cell when the expression of these genes is suppressed in response to different stimuli, for example a point mutation or a genetic cause; hence cell growth is no longer limited. DNA repair genes are not directly responsible for cancer formation, however, since they are the care takers of the DNA, their absence or inadequate function will lead to instability in the genome and thus leading to a higher chance of cancer formation. A lot of research on telomerase expression resulted recently in the discovery that telomerase is present in almost all carcinoma cells; therefore, telomerase could have a great diagnostic and maybe even therapeutic value.[4]

The transcription factor Nuclear Factor kappa B (NF-kB), will be discussed shortly to give a quick impression of the complexity and interaction chains involved in the onset of cancer. The NF-kB complex in the cytoplasma of the cell is usually bound to the IkB family making it impossible for NF-kB to travel to the cell nucleus and bind to the DNA for further action. Many different signals, such as growth factor and hormones, can result in the activation of IkB kinase responsible for phosphorylation of IkB and

thereby releasing NF-kB dimers. These dimers are then translocated to the nucleus where they bind to the kB location in the promoter or enhancer region of the target genes controlling the expression of this gene. When NF-kB is activated, the transcription of many genes is induced. Moreover, NF-kB seems to be a key mediator in inflammation, tumor onset and growth and the formation of blood vessels. NF-kB activation is also known to be associated with numerous types of cancer.[5] This example should give us an understanding of how important it is to reveal the function of these molecular factors in cancer and by further exploring some of these processes. This is exactly where the field of molecular imaging has been indispensible. By revealing these biological processes step by step, each discovery is a step closer to better cancer diagnostics and treatment.

One can also think about the biological processes of metastatic cells, which involve numerous genes and proteins. The journey of metastasis is nonetheless not easy for a cancer cell. The estimation is that around 0.01% of the tumor cells will be able to depart from the original site, survive the blood stream, attach to a suitable tissue and manipulate normal cells for the malignant transformation. However, once a cancer cell is able to metastasize, the new colonies are more resistant to the standard treatment than the original tumor due to their genetic alternations.

To overcome this problem research focuses on different theories of metastasis. One can see cancer for example as an inflammatory disease that uses immune cells for its spread and therefore the metastasis can be limited by the inhibition of the immune system. Or one can see metastasis as an embryonic process and the traveling cancer cells are in fact cancer stem cells that use the properties of a stem cell to migrate.

Recently, individual tumor cells were detected in the blood stream of patients with early-stage cancer, suggesting that the onset of metastasis might even take place in the beginning of the cancer formation. This would open the doors for early-stage cancer detection by simple blood assays.

Nevertheless, the facts that some tumor cells need years to spread whereas others go out immediately and that metastatic tumor cells look different in every organ, remain a barrier for early detection and treatment. Besides, what if the cancer cells that are not killed by the chemotherapeutic obtain mutations, which transform them into even more resistant killers?[6]

2. GLIOMA

Gliomas account for 31% of all tumors in the Central Nervous System (CNS) and for 78% of all CNS malignancies (www.Cbtrus.org). Malignant gliomas are classified as astrocytoma, oligodendroglioma, or oligo-astrocytoma and histologically graded as WHO grade II, III (anaplastic) or IV.[7] Grade II tumors are associated with a survival time of 5 to 15 years, while grade III gliomas often predict a survival time of less than 3 years. Glioblastoma Multiforme (GBM, or astrocytoma grade IV) is the most malignant of all glial tumors and has an extreme poor prognosis, with an average 5 years survival of only 3.3% and the majority of patients dying within a year. Without treatment median survival is 4-6 months.[8] The characteristics of this malignancy include uncontrolled cellular proliferation, invasiveness with both long root like processes and single invasive cells, areas of necrosis, and extensive angiogenesis.[9, 10] Furthermore, the GBM cells are resistant to apoptosis, and possess multiple genetic alterations.[11] Primary GBM occurs de novo, without a pre-existing less malignant precursor lesion, and comprises over 90% of the GBM cases. Secondary GBM occurs through progression of a low grade astrocytoma or anaplastic astrocytoma and generally occurs in younger patients.[12]

The current standard of care treatment for GBM consists of surgical macroscopic debulking of the tumor mass, followed by both radiation and chemotherapy.[13, 14] Progression free survival in the first 6 months appears to be directly related to the amount of tumor mass removed, with a more extensive resection corresponding to a better outcome (41 vs. 21%).[15, 16] Combined treatment with radiation and temozolomide further increases median survival to 12-14 months, which is significantly better than the results achieved with radiation alone (median survival: 9 months).[8, 15, 17] However, regardless of treatment most patients die within a year from new secondary tumor foci forming within 2 cm of the resected area.[18, 19]

2.1 Factors complicating GBM treatment

Evidently, GBM is difficult to treat. Average survival increased with months instead of years, while researchers worldwide are working hard to find a cure. The aggressive

behavior of GBM tumor cells is caused by an array of tools that are specifically designed to escape eradication. The distinct tumor heterogeneity, the ability to escape the cellular immune response, the resistance to therapy, the interaction between the tumor cells and the microenvironment, and the inability of treatment to reach all tumor cells make GBM such a challenge to treat.

2.1.1 Tumor heterogeneity

One of the hallmarks of GBM is its heterogeneity. Cells differ in morphology, behavior and genetics[20] and consequently it is very difficult to grade the tumors, measure response to therapy and understand the mechanisms of resistance. Not one GBM tumor is similar to another. Primary and secondary GBM appear morphologically the same, but genetically, differences are profound.[21] It is thus unlikely that one standard therapy aimed to cure all tumors diagnosed as GBM can be developed.

Necrosis is one of the features of GBM and is thought to play an important role in the development of the heterogeneity of the cell population. First, hypoxia is likely to appear in tumor regions where metabolic demands exceed the supply or as a result of thrombolytic events that are often seen in glioma patients. As a result, migratory genes are triggered and cells start to move away from the hypoxic site, whereas necrosis ensues in the hypoxic center. A lining of palisade cells develops around this necrotic core, expressing an abundance of angiogenic and growth factors and thereby facilitating angiogenesis and tumor proliferation.[22, 23] At the same time, a clonal selection takes place selecting highly malignant tumor cells that are resistant to apoptosis by inactivation of p53.[24] The selection of more highly malignant cells may then again lead to a higher metabolic demand, causing a vicious cycle of hypoxia, necrosis, selection and proliferation; resulting in a very heterogeneous population of cells, which are highly resistant to conventional therapy. Therefore, necrosis is a very powerful predictor of a bad outcome.[10]

2.1.2 Tumor invasion in the microenvironment

Gliomas are known for their tendency to infiltrate the surrounding brain parenchyma, which makes it very difficult to rely on locally applied treatment, like surgery. After initial surgery, tumor reoccurrence usually takes place within 2 cm of the original tumor site, suggesting that at the time of surgery individual cells already invaded the surrounding brain tissue. This is one of the major challenges in GBM treatment and makes a better understanding of both tumor cell biology and tumor microenvironment highly desired.

Tumor invasion is a very intricate process involving a combination of the ability to migrate (cell motility) and the ability to modulate the extracellular matrix (ECM). These abilities are present in both low and high grade gliomas, suggesting they are acquired early in tumorigenesis.[9] Preferential patterns of migration can be discerned, including migration along the white matter tracts, around neurons in the gray matter (a phenomenon known as perineural satellitosis), perivascular growth and subpial spread.[9] These patterns suggest the existence of some sort of tropism or a restricted ability of GBM to invade specific regions between certain cell combinations and also show that interactions with the tumor microenvironment play an important role in the process of invasion.[25]

To facilitate invasion, GBM cells display a wide array of tools. First, a variety of proteases such as cysteines, serines and matrix metalloproteinases (MMP's) is secreted by the tumor cells to degrade the ECM in order to allow migration, and to remodel the ECM in a way that facilitates tumor growth.[26] Expression of these proteases increases with tumor grade. Further, an increase of integrin receptors can be observed, facilitating the interaction of the cells with the ECM molecules and thereby modifying the cell cytoskeleton towards locomotion. Kinases such as the cytosolic Focal Adhesion Kinase (FAK) are activated by epidermal growth factor receptor (EGFR) which in turn activates downstream pathways involved in proliferation, survival and migration.[27, 28] Growth factors as fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet derived epidermal growth factor (PDEGF) and their receptors are upregulated and promote both proliferation and migration. EGFRvIII mutation is often

found in GBM and is known to upregulate expression of genes responsible for MMP and collagen production, thereby further facilitating invasion.[29]

The final tool GBM cells display in their attempt to remodel the ECM into an optimal niche for growth and development is the creation of a zone of local immune suppression. Immunosuppression is facilitated by cell-to-cell contact and by the secretion of various cytokines. This strategy leads to T cell activation inhibition and the initiation of T cell apoptosis, and thereby prevents the immunesystem form actively attacking the tumor cells.[30]

2.1.3 Angiogenesis in GBM

In order to adapt to hypoxic conditions once the tumor mass increases, GBM cells release pro inflammatory agents. Also, GBM cells have the ability to transdifferentiate in tumor derived endothelial cells (TDEC).[31] These TDEC are capable of forming vascular structures within the tumor, reestablishing oxygen and nutrient flow and making GBM one of the most vascularized tumors. However, anti-angiogenesis treatment with anti-VEGF receptor inhibition does not seem to affect the TDEC's.

2.1.4 Blood Brain Barrier

Since extra-neural spread of GBM is very rare, the most convenient method of treatment would be local in the CNS. However, the delivery of therapeutics is challenging due to a natural filtering mechanism, the blood-brain-barrier. Most chemotherapeutics are unable to cross this barrier, or are cleared very rapidly out of the brain extracellular space. The integrity of the blood-brain-barrier varies per region and this affects the locally available drug concentration.[32] GBM cells are capable of pumping out drugs after uptake, by means of P-glycoprotein and other pumps.[33] The high intratumoral pressure further complicates delivery of therapeutic agents. Damage to healthy brain tissue, due to the limited intracranial space, is another point of concern.

2.1.5 Cancer Stem Cells

The “old” stochastic model on tumor growth proclaims that all cells in a tumor are biologically equivalent and are able to initiate or drive tumor formation, due to accumulation of mutations.[34, 35] In contrast, the Cancer Stem Cell (CSC) theory suggests that a rare population of tumor cells is responsible for tumor growth, resistance and recurrence.[36] These cells are named Cancer Stem Cells because of their “normal stem cell” like properties; they share important characteristics with stem cells, including their ability of limitless self-renewal and differentiation.[37] They are capable of generating a diverse population of cells, both tumorigenic and non-tumorigenic, present in tumors. They seem to exclusively drive tumor growth and to give rise to a diverse progeny.[38] Once implanted in immunogenic mice, CSC are capable of generating a photocopy of the original malignancy of which they were extracted.[39] Both models are depicted in figure 1.

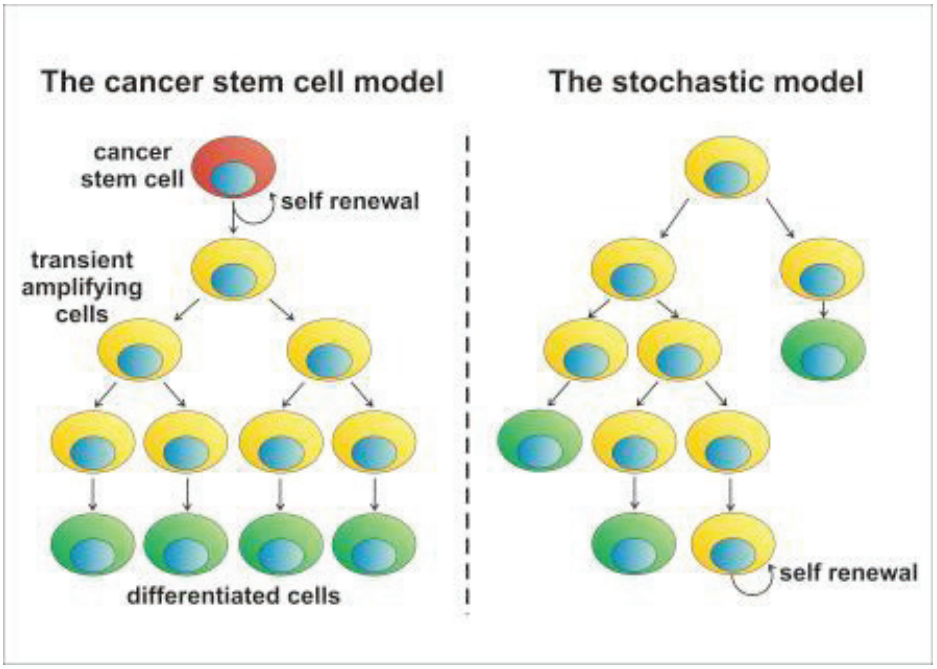

Figure 1. Model of cancer theories. The CSC theory suggests a strong hierarchal pattern within the tumor. Cancer stem cells are the only cells capable of self-renewal and proliferation. They initiate and drive tumor growth. The stochastic model describes tumor

growth as a random process. Accumulation of genetic alterations and mutations drives tumor growth and all cells can contribute to this process. Red: CSC. Yellow: transient amplifying cells. These cells are slowly maturing and lose their ability of self-renewal along the way. Green: fully differentiated tumor cells that are no longer capable of self-renewal. Image adapted from www.eurostemcell.org

The CSC theory implies that indiscriminate killing of all cancer cells may be an inefficient and ineffective way to treat cancer, since it is not targeted to eliminate the few CSC that actually drive the cancer. In this scenario the treatment will kill the proliferating "innocent" cell population, and since these cells consists of the majority of the tumor, treatment will seem effective. However, CSC are known to be very resistant to chemo- or radiotherapy due to their stem cell-like properties and their relative quiescence, and will remain at the tumor site (unless surgically removed), eventually causing a relapse (Figure 2).[33, 40, 41] Selective targeting of CSC might be a better approach.

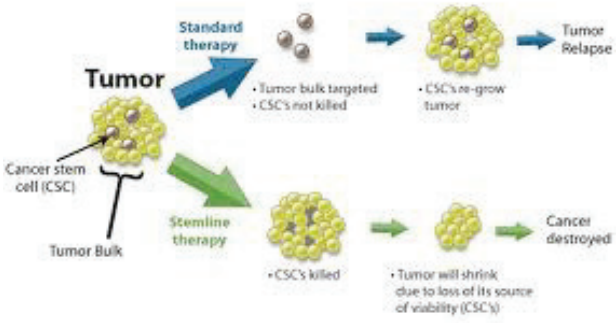

Figure 2 The effect of two different therapeutic approaches on CSC. If standard therapy is used, CSC will escape elimination and start the process of tumor initiation and proliferation all over again. Tumor relapse seems inevitable. If however, tumor therapy is aimed at CSC directly, CSC will die and the remaining tumor bulk, with no tumorigenic capacities of its own, will shrink and disappear, and no recurrence will occur. Image adapted from Lui et al. Cancer Research, 2011.

In GBM, glioma stem cells (GSC) have shown similarities to normal stem cells and progenitor cells, expressing the markers CD133 and Nestin.[37] This leads to concern of toxicity when using these markers to design drugs targeted to GSCs. Since glioma stem cells produce vascular endothelial growth factor (VEGF), promoting

angiogenesis, and since they appear to need a vascular niche for optimal functioning,[42-44] the use of anti-angiogenic therapy to inhibit glioma CSC functioning might be a better strategic approach. There is ongoing discussion over the exact role of CSC and their characteristics,[45] but nonetheless CSC are believed to play an important role in GBM tumor initiation, progression and angiogenesis, making GBM so complicated to treat.

2.1.6 Genetic alterations and resistance to apoptosis

Resistance to apoptosis of GBM cells is very common. Genetic alterations that upregulate oncogenes and inactivate tumor-suppressor genes (including retinoblastoma protein (RB) and p53) are found in the majority of GBM cells.[46, 47] This, combined with the natural selection of highly malignant clones, and the stem cell-like properties of a subgroup of the GBM cells, make that GBM cells are highly resistant to apoptosis.[33] In addition, GBM cells express a variety of molecules affecting both intrinsic and extrinsic apoptotic pathways. They secrete soluble decoy death receptors aimed at the natural defense mechanisms and often the Bcl2 family of anti-apoptotic genes is upregulated. Crosstalk between the anti apoptotic pathways further contributes to the difficulty of finding an effective treatment.

3. WHAT ARE THE CURRENT STRATEGIES FOR TREATING GBM?

3.1 Current treatment

The National Comprehensive Cancer Network states that standard treatment of GBM consists of maximal surgical removal of the tumor mass, 6 weeks postoperative radiotherapy, and concomitant systemic chemotherapy with temozolomide followed by 6 months of adjuvant treatment with temozolomide (NCCN guidelines version 2.2011: CNS Cancers, www.nccn.org).[48, 49] Advances in both surgical and imaging techniques permit safer and more extensive removal of the tumor, but due to the highly invasive nature of GBM surgery is not intended to be curative. The prognosis after recurrence is very poor, and recommendations for adjuvant treatment strategies are ill defined. Current options include surgery with or

without camustine wafer placement (if the recurrence is local), radiotherapy, chemotherapy, anti-angiogenic agents (monoclonal antibody bevacizumab), or experimental therapies (www.nccn.org). Surgery seems to prolong survival up to a limited degree. The benefits of repeated radiotherapy remain unclear.[50-52] Due to toxicity to normal cells, high enough doses can't be delivered. Resistance to chemotherapy can be overcome by changes in dose regimen and by combining temozolomide with the cyclo-oxygenase 2 (COX-2) inhibitor rofecoxib, leading to an anti angiogenic effect.[52] Interestingly enough, not all anti-angiogenic agents seem to enhance the efficacy of temozolomide treatment. Combined temozolomide and bevacizumab regimens resulted in inferior outcomes than seen after treatment with bevacizumab or temozolomide monotherapy.[53] This may be partially explained by many patients with CNS tumors however require dexamethasone or anti epileptic drugs, which in combination with temozolomide or other anti cancer agents, may lead to drug-drug interactions with a reduced efficacy and an increase of side effects.[54]

Genotyping for personalized medicine is slowly starting to influence treatment options. 60% of GBM tumors with chromosomal 1p loss respond to a chemotherapy regimen of PVC (procarbazine, CCNU and vincristine) combined with temozolomide, while 'regular' GBM tumors are not sensitive to this specific regimen. GBMs with EGFR amplification rarely respond to chemotherapy at all. O6- methylguanine DNA transferrase or MGMT, a DNA repair enzyme that protects cells from damage caused by ionizing radiation and alkylating agents, is another powerful molecular predictor.[55, 56] The MGMT promotor is methylated in 40 to 45% of GBMs, which means that cells are unable to properly repair DNA damage.[57, 58] MGMT methylation is currently the strongest predictor of outcome and benefit of temozolomide treatment.[59] Simple genotyping assays screening patients for chromosomal 1p loss, EGFRIII mutation and MGMT promotor methylation can therefore not only increase quality of life (only exposing those patients to treatment that have a high chance of good response), but can further result in higher survival rates, since no time is wasted on the 'wrong' type of therapy.

Since GBMs are highly vascularized tumors, **anti angiogenesis strategies** have received a great deal of attention. VEGF expression levels correlate with tumor

malignancy levels and many angiogenic factors are secreted (VEGF, PDGF, fibroblast growth factor 2 (FGF2), Hepatocyte Growth Factor).[60] Monoclonal antibodies directed against VEGF or its receptor (e.g. Bevacizumab) are FDA approved and currently in use, although treatment did not prove to be more effective than standard therapy (www.nccn.org)[61] However, quality of life seemed to improve.[62, 63] Side effects related to toxicity, resistance, and progression to a more invasive type of tumor are reported. Other strategies including small molecule inhibitors (Cediranib) designed to inhibit VEGFR2 Tyrosine Kinase activity, or soluble decoy receptors identical to VEGFR1 (Aflibercept) are currently under investigation in clinical trials.[64, 65]

3.2 New foci of research

Due to the limited success of therapies discussed above, new foci of research have emerged. As discussed earlier, GBM tumors are highly heterogeneous, display all kinds of anti-apoptotic escape routes, suppress the immune system, invade the surrounding parenchyma with unmatched aggressiveness and possess a whole array of tools to rearrange the extra tumoral environment to their advantage. Integrins, the cell surface receptors responsible for cell adhesion to the ECM, are known to play a crucial role in the recruitment of the ECM by activating intracellular pathways responsible for cell survival, migration, and angiogenesis in both GBM cells and cells in their direct environment (fibroblasts, vascular endothelial cells, bone marrow derived cells).[66] Further, interaction between GBM cells and ECM molecules results in modification of the GBM cytoskeleton and locomotion. In a Phase II clinical trial, Cilengitide, a synthetic cyclic peptide that blocks the binding of integrin to its receptors, showed moderately positive results.[67] This drug is also being tested in combination with other anti cancer agents (www.clinicaltrials.gov).

Other targeted therapies aimed at disrupting the interactions between GBM and ECM include receptor tyrosine kinase inhibitors (blocking the activation of intracellular pathways associated with cell proliferation and migration) and small non-coding RNA's, which are used to inhibit cell to cell signal transduction and activation of stem cell pathways. The latter approach is believed to directly target cancer stem cells and, since this population is thought to be the driving source of tumor

proliferation and metastasis, should result in less aggressive behavior of the tumor as a whole. Unfortunately, so far the results of these strategies have been poor, with no survival benefit in GBM patients.

The fact that many of the single agent targeted therapies seem to fail is most likely attributable to the complexity of the interactions between GBM and ECM and to the crosstalk between the different intra- and extra cellular pathways, allowing tumor cells to overcome interventions and to escape cell death over and over again. The relatively low number of patients diagnosed with GBM makes recruitment for clinical trials and testing of new agents even more problematic. Further advances in surgery, genomics, proteomics, genetics and imaging modalities will be needed to get more insight in GBM tumor biology and to find diagnostics, predictive biomarkers, and targeted strategies to treat GBM successfully.

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