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Using REMBRANDT to Paint in the Details of Glioma Biology: Applications for Future Immunotherapy

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Additional information is available at the end of the chapter

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1. Introduction

Microarray technology developed in the late 1990's allows one to simultaneously analyze the entire transcriptome of a given population of cells at a single time. It is essentially a microchip with each spot containing about a picogram of DNA immobilized at defined locations. This DNA captures mRNA or cDNA from defined cell populations (tissue, cell line, *etc*) in a quantitative manner. This technique allows complex patterns of global gene expressions to be quickly and easily detected between two specimens. Microarrays were initially used to describe cell cycle patterns of *Saccharomyces cerevisiae* [1]. Its powerful potential was quickly recognized and applied towards cancer. The first successful application of this technology was to show that diffuse large cell B lymphomas had two different subtypes. One subset of this cancer was easily treatable with chemotherapy; whereas, the second phenotype required a more aggressive therapy [2].

Microarray analysis of human GBM quickly revealed that this cancer could be divided into three different subtypes: neural/pro-neural, mesenchymal and classical GBM [3]. Each subset has its own unique profile of gene expression along with a unique survival pattern. The microarray data also represents a composite of the tumor and the surrounding non-cancerous cells that are involved in the microenvironment including host immune cells. Microarrays have also recently provided possible mechanisms that GBM have used to avoid the effects of anti-angiogenic therapies [4]. Thus, this *in silico* technology offers new strategies to target various cancers in still many unknown ways.

In 2005 the National Cancer Institute and the National Institute of Neurological Disorders and Stroke (NINDS) initiated the REMBRANDT (REpository for Molecular BRAin Neoplasia DaTa (<http://rembrandt.nci.nih.gov/>) database. This informatics venture originated from the leadership of Dr. Subhashree Madhavan (NCI Center for Bioinformatics) and Dr. Howard Fine (Glioma Molecular Diagnostic Initiative at the Center of Cancer Research). Currently this research portal molecularly characterizes a large number of primary brain tumors and correlates this gene expression, copy number data with patient survival. At this time, there are several active collaborators who are supplying information into this database: Henry Ford Institute (Detroit), Johns Hopkins University (Baltimore), National Cancer Institute-Neuro-Oncology Branch, National Institute of Neurological Disorders and Stroke (NINDS), M.D. Anderson Cancer Center (Houston), Mofitt Cancer Center (Tampa), Thomas Jefferson (Philadelphia), Univ. of California, Los Angeles, Univ. of California, San Francisco, Univ. of Pittsburgh Medical Center, and Univ. of Wisconsin Carbone Cancer Center (Madison). At the time of writing this chapter 568 brain cancer specimens are available for gene expression analysis, while 552 patients were analyzed (May-Aug 2012) for copy number studies using REMBRANDT version 1.5.5. Thus, we have a relatively large database for which we can draw good information to determine the usefulness of targeting any given gene of interest towards a given brain cancer.

Van der Bruggen, *et al.*, [5] compiled a listing of various tumor antigens that have been found within various human cancers. Tumor antigens can be defined as either being tumor-specific or tumor-associated. These tumor-associated antigens are composed of mutations, shared tumor-specific, differentiation and over-expressed antigens. Tumor specific antigens are actually quite rare. The epidermal growth factor receptor variant III (EGFRvIII) is an example of a glioma-specific tumor antigen. Most antigens used for cancer therapy are shared antigens, in which the cancers over-express these proteins. By this definition most antigens should be described as cancer-associated antigens, as opposed to cancer-specific antigens. When tumor antigens are discovered, either by molecular techniques or using immunological techniques, only a handful of tumor cases are actually evaluated to generate the initial scientific report. Hence the real scope of the true expression pattern was usually limited due to a small sampling size of the initial report. Analyzing large databases are perhaps the best way to validate the universal nature of any tumor antigen.

Traditionally, there are antibody-based or cell-mediated-based antigens. Back in the 1970's-1980's, antibody responses were viewed as the best way to treat tumors. These antibodies need to be specifically designed to bind to cancer-specific cell surface proteins. Antibodies have been detected coming from glioma patients (PHD finger proteins-3 and -20 were identified [6, 7]. When these were evaluated by REMBRANDT, there were only 1 and 5 patients who over-expressed these respective transcripts and there were no statistical significance. Many antibodies were designed against glioma cells. Antibodies targeting internal antigens will obviously not be clinically applicable, since these antibodies' binding regions can't access their targeted intracellular epitopes. Antibodies with 150,000 dalton molecular weights can't penetrate deeply into intracellular tumor cells due to their inability to cross cell-adhesion junctions between adjacent cells. Peripherally located tumor cells usually bind the most

amount of antibody, leaving the more internally located ones less affected. Single chain variable antibody fragments (scFv) are being developed out of monoclonal antibody technology can penetrate deeper into tumor beds, since they have lowered molecular weights (about 15-20 kd). ScFv targeting EGFRvIII, MRP3, TRAIL and c-Met have been used in preclinical models of human gliomas [8-11]. But just like regular antibodies, the scFv will undoubtedly predominantly bind to peripheral tumor cells. The clinical efficacy of scFv still has not been firmly established.

In contrast, internal and external antigens can be processed by intracellular proteasome using a universal ubiquitization pathway. Eventually some of these antigenic peptides are presented on the cell-surface of major histocompatibility (MHC) molecules, provided they have the right affinity and the right concentration. So once the correct anti-cancer T cells are activated towards these peptides, the T cells can respond to multiple tumor cells. The caveat to this approach is that many tumor cells down-regulate their MHC expression, making themselves “invisible” to the T cells. The use of dendritic cell (DC)-based vaccines to generate endogenous T cells *in vivo* seems to show that T cell-induced therapies are a better way to treat gliomas than humoral approaches [12]. Prins, *et al*, [13] showed that DC-based therapy using the autologous tumor to prime the T cells works better against the mesenchymal type of GBM than the other two subsets. So the identification of the right tumor antigens that glioma cells possess is the Holy Grail of tumor immunotherapy.

2. Hallmarks of cancer

In 2012 Hanahan and Weinburg updated their classic “Hallmarks of Cancer” paper where they described the original six Hallmarks (resisting cell death, sustained proliferation, evading growth suppression, activating invasion and metastasis, enabling replicative immortality and inducing angiogenesis) with two more emerging Hallmarks (deregulating energetic and avoiding immune destruction), along with two enabling characteristics (genomic instability and mutation and tumor promoting inflammation) [14]. Hanahan and Weinburg pointed out that various anti-cancer therapies currently being developed actually target one of these possible ten Hallmarks of Cancer. Each of these drugs is designed to be very specific and only affect a certain cancer pathway. Hence these drugs should avoid any unwanted side effects. One obvious down-side to this way is that cancers are very resourceful and can quickly mutate to avoid the actions of this drug.

They also speculated that if you design a drug that targets two or more of these pathways, then that drug should then theoretically be more potent than a drug that targets a single “Hallmark”. We think it is possible to simultaneously target multiple “Hallmarks”. A whole tumor cell vaccine which targets a wide variety of possible “Hallmarks” is possible.

Figure 1 shows some representative glioma antigens that are known and can induce immune responses against seven of the “Hallmarks”. So the true power of tumor vaccines using whole cells as the source of the vaccine might be that it targets multiple proposed Hallmarks of Cancer

at once. So finding newer antigens that might additionally target different Hallmarks could also improve immunotherapy.

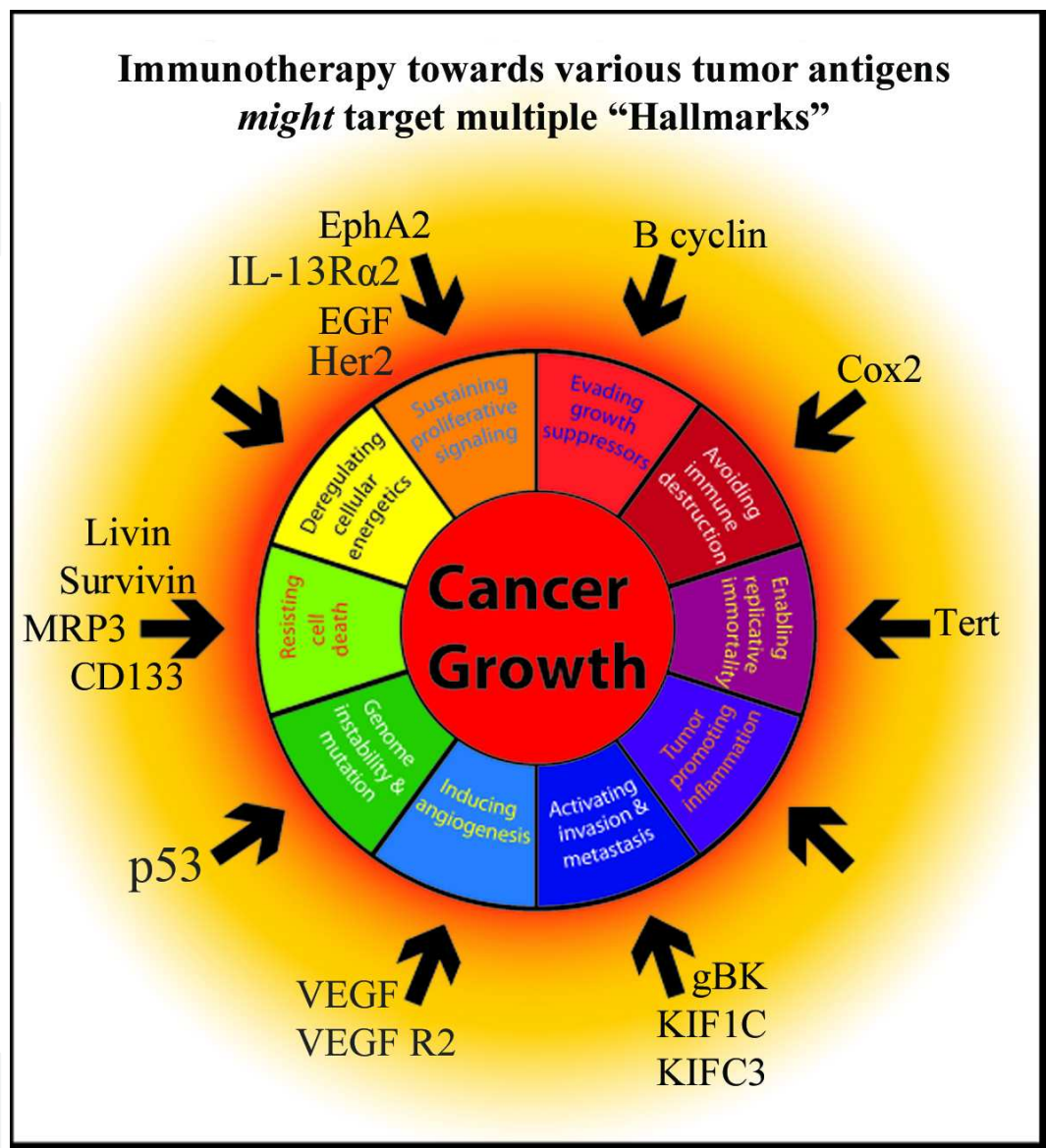


Figure 1. Whole tumor vaccines can target multiple Hallmarks of Cancer. Listed are some glioma tumor-associated antigens which can be classified under the various categories of the Hallmarks of cancer.

3. Current glioma associated tumor antigens: How well do our current tumor antigens fit the survival data of GBM?

REMBRANDT is quite easy to use. One can access the data of all glioma types or limit the selection of brain cancers to GBM, mixed gliomas, or oligodendroglioma. At the gene expression level, one can concentrate on over-expression that is 2.0-fold better than control samples,

or under expression < 0.5-fold expression, or an intermediate expression pattern. For this chapter we will be examining the universal nature of all glioma types. If readers want more specific brain cancer subtypes, they can easily study the various subsets of brain tumors on their own using REMBRANDT.

Previously, many tumor antigens used for glioma immunotherapy have been described [15-20]. A good tumor-associated antigen is one that is found in a large number of human glioma patients and has many different epitopes, so that many different T cell clones can respond to it, regardless of the major histocompatibility complex haplotype. The targeted antigen should also be associated with a poor prognosis. Therefore properly targeting this antigen interferes with some key glioma cell function; i.e., cell division, growth factor signaling, migration and other processes of the Hallmarks of Cancer. Improved patient survival if a successful therapy was mounted against this tumor antigen, should also eliminate those antigen-positive cells. By changing the tumor population from an antigen-positive one, to an antigen-negative one, overall survival should improve, if that antigen/protein was indeed critical to glioma biology. Even at this time, we still don't precisely know what the best glioma-specific antigens are to use for immunotherapy, except by doing expensive clinical trials. The other theoretical possibility, is that the antigen must be found on every tumor cell. In which case, every antigen-positive cell needs to be eliminated. In which case, every antigen is created equally. This question will eventually need to be answered, before immunotherapeutic cures can become a reality. Our own speculation is that not all glioma-associated antigens are equally useful.

When the currently known glioma-associated tumor antigens are subjected to REMBRANDT analysis, some of these antigens do seem to show a good inverse correlation with survival. Having more of the antigen does seem to correlate with a poorer survival in a dose-dependent manner. Figure 2 shows a representative illustration of this effect using the well-known glioma-associated antigen, IL13R α 2. There were 121 patients (red) who possessed elevated IL13R α 2 mRNA levels (21% of all glioma patients), while 115 (20% of all gliomas examined) had down-regulated IL13R α 2 expression (green). The more IL13R α 2 mRNA the patient's glioma had, the poorer the prognosis of these patients was. This data can be considered as a dose response experiment. By the Log-rank test, provided by REMBRANDT, these values were quite statistically significant from the total 568 glioma patients (blue) that were examined. Thus, this tumor antigen should be good one to target for a subset of glioma patients, since the lower the expression of IL13R α 2 lead to better patient survival. Other very good glioma-associated antigens are: EphA2, FABP7, FosL1, MRP-3, NR2E1 and podoplanin (Table 1).

Some antigens such as survivin/Birc5, CD133, her2 and Sart-2 were considered good antigens in that either their over-expression lead to poor survival or their under-expression had a better prognosis. This observation could be that some samples didn't have sufficient samples to provide a statistical significant value. WT1 also seems to be a good target. In an early clinical trial using WT-1 peptide vaccinated patients, there was some survival benefit for the first 100 weeks after the vaccination [21]. Thus, an early test of our hypothesis seems at first glance to be a viable one.

Very Good Antigens	Good Antigens	Poor Antigens
BCAN,CHI3L2, EphA2,FABP7, FosL1, IL-13Ra2, MRP3, NR2E1, PDPN, YKL-40/CHI3L1	B3GALNT/Galt3, CD133, Her2, IGF2BP3, Sart-2, Survivin, WT-1	Art-1, Art-4, ARF4L/ARL4D, CLIP2, CSPG4,EphB6, EZH2, Gnt-V, GP100, KIF1C, KIF3C, HNRPL, livin, Mage-A1, MELK, NES, NLGN4X, NRCAM, PHF-3, PHF-20, Prame, PTH-rP, PTPRZ1, Sart-1, Sart-3, SLC01C1, Sox-2, Sox-6, Sox-10, Sox-11, SSX2, TNC, Tert, Trp-2/DCT, Tyrosinase, Whsc2

Very Good Antigens: both over-expression predicts shorter patient survival and under-expression leads to longer survival rates. Statistical significance reached by REMBRANDT.

Good Antigens: one value (over-expression or under-expression) achieved statistical significance.

Poor Antigens: Neither over- nor under-expression correlated with patient survival and had any statistical significance.

Table 1. Currently known Glioma-associated antigens and their predicted usefulness against all gliomas as predicted by REMBRANDT.

Unfortunately, most reported glioma-associated antigens such as Sox2, Sart3, *etc* do not seem to have any prognostic value. Their expression profile does not indicate any positive or negative effect. From this we would conclude, even though the glioma cells carry this tumor antigen, and *in vitro* experiments suggest that CTLs can kill tumor cells bearing this specific antigen, this antigen does not appear to have any selective or predictive advantage. Therefore even if a robust immune response were mounted *in vivo* by some vaccination or therapeutic process, chances are this response will not produce any demonstrable effect, unless all of those antigen-positive tumor cells were eliminated. Therefore, just because CTLs can be generated against tumor cells doesn't necessarily mean it will produce any clinical response, unless every tumor cell was eliminated. Killing every tumor within any cancer is highly doubtful.

In conclusion, some of our currently known tumor-associated antigens found in human glioma are still good ones to target by therapeutic interventions including immunological ones, while others probably will not be that much in helping eliminate this cancer. Searching out new target antigens is still a vital goal of immunotherapy and new strategies to find more appropriate antigens are required. This may be where REMBRANDT can come in quite handy in developing intelligent rationales for designing better targets for future immunotherapy (see below).

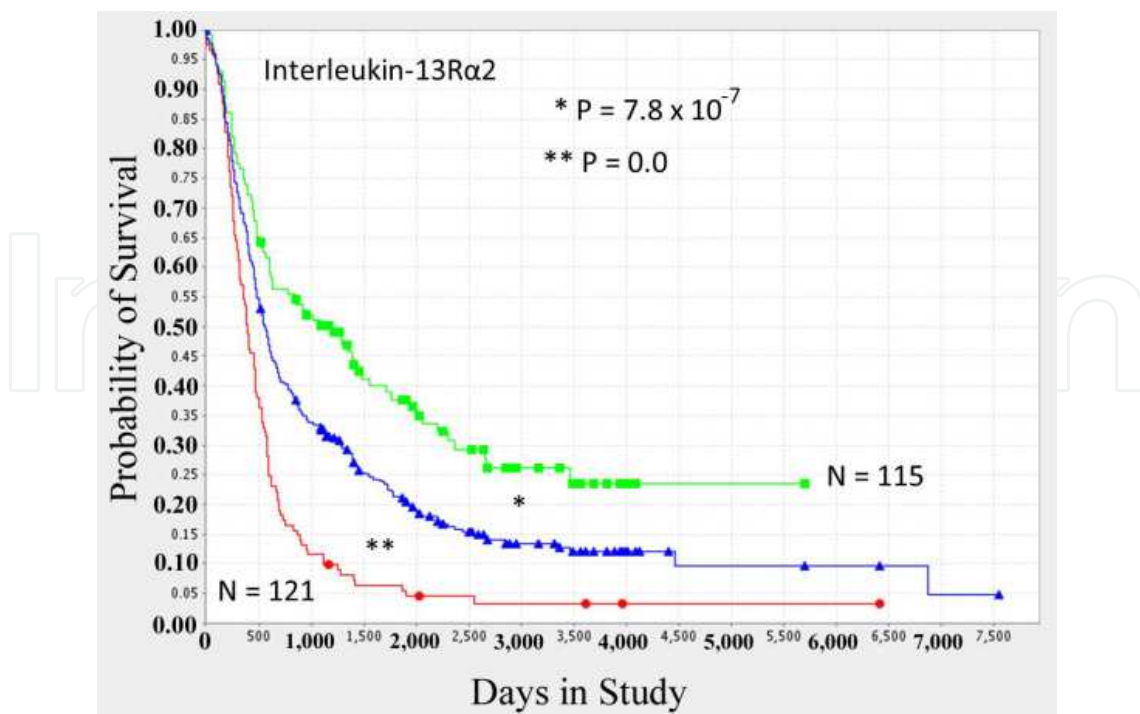


Figure 2. REMBRANDT survival of all gliomas showing differential IL13Rα2 expression. The blue symbols show the survival of all glioma patients. The red lines show the survival of IL13Rα2 over-expressing gliomas. While the green symbols show the patients that survived with a low expression of IL13Rα2. Both populations were statistically significantly different (asterisks) from the majority of all gliomas (blue).

4. Cells of the immune system involved in glioma

The immune system plays an important role in combating a wide variety of pathogens ranging from viruses, bacteria, fungi, parasites and others. Cancers can also be considered a type of pathogen that the body must rid itself of on a routine basis. In the mid-1980's, CD4⁺ T cell clones were classified into various subsets of T helper cells, Th1 cells produced cytokines that stimulated cell-mediated functions, while Th2 cells produced humoral immune responses [22]. New CD4 T helper subsets are still being discovered. Th9, Th17, Th22, Tfh and Treg are populations recently described in the last several years. Each subset seems to have its own niche with regards to certain immunological functions controlling certain pathogens or antigens. Besides CD4⁺ T cells, CD8, NK, and $\gamma\delta$ T cells all play roles in tumor immunity.

Figure 3 shows these various subsets that are currently known along with some of their postulated effector cytokines. By REMBRANDT, we tried to analyze these different T cell subtypes. We examined their respective cytokine(s), possible effector molecules or putative transcription factors that control the transcription of these cell types.

REMBRANDT surprisingly revealed that CD4 expression had a bad prognosis for glioma patients. Over-expression of CD4 had a statistically significant poor survival prognosis, while an under-expression trended towards better survival, but this last condition was not consid-

ered significant ($p=0.10$). Naïve $CD4^+$ T helper cells can give rise to numerous subtypes of $CD4^+$ T cells: Th1, Th2, Th9, Th17, Th22, Tfh, and Treg. Thus, the sum total of all these $CD4$ subsets represents this poor overall survival.

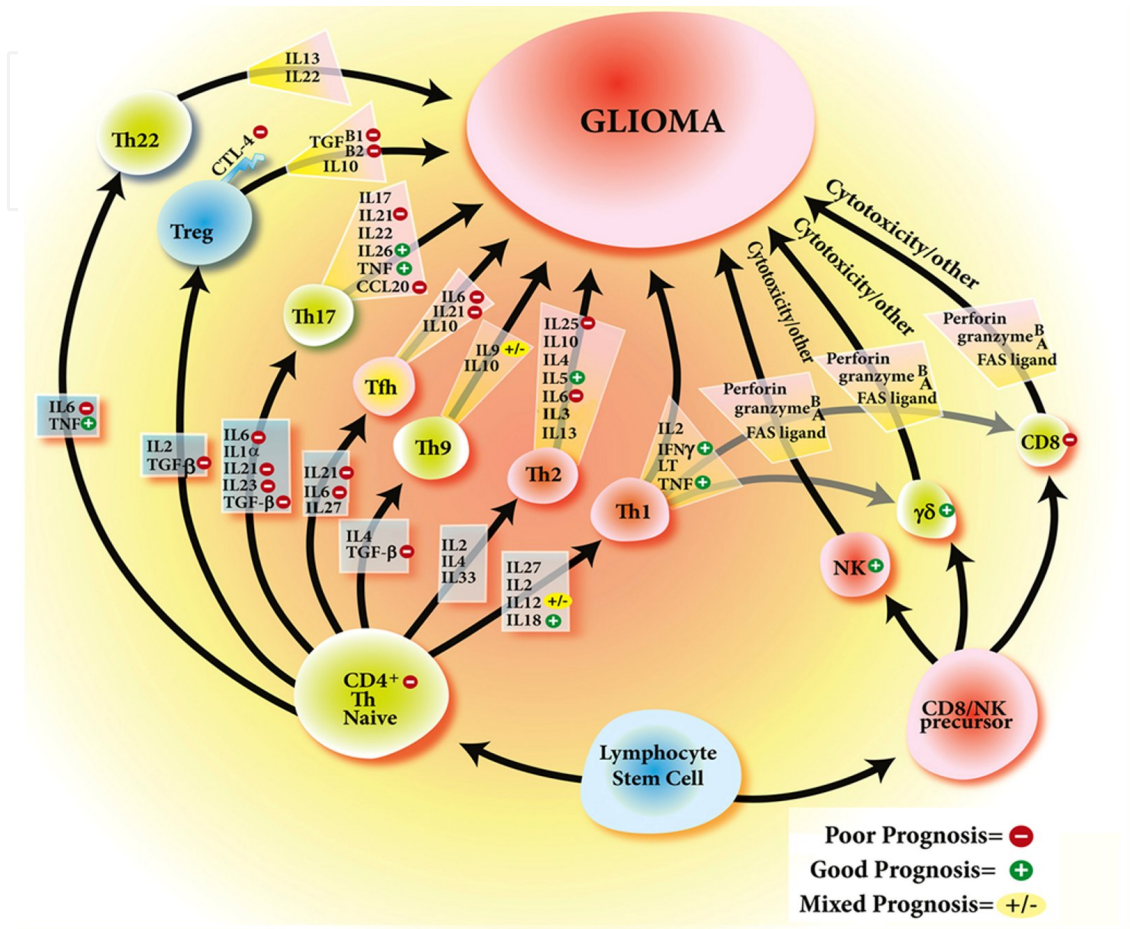


Figure 3. The cells of the Immune system and how they mediate some of their functions. Lymphocyte precursors give rise to either $CD4^+$ or $CD8/NK$ precursors. As a result of T cell receptor engagement along with the presence of cytokines shown in the rectangles can give rise to various T helper subsets. After the Th subsets are restimulated they can then release various cytokines as shown in the polygons. These cytokines can either affect the glioma or the cells in the local microenvironment. $CD8/NK$ precursors can differentiate into either NK, $\gamma\delta$, or $CD8$ T cells. These cells can either kill tumor cells directly or via other undefined pathways.

It has been generally acknowledged that Th1 cells produce cell-mediated immunity that results in effective anti-tumor immune responses. Th1 cells tend to release cytokines such as IL2, IFN- γ , TNF α and LT, that either work directly on the tumor or indirectly by stimulating effector anti-tumor cells, CTLs, NK, macrophages, etc. REMBRANDT suggested the expression of IL2 portrays a slightly better survival when these cytokines were elevated in patients but these were not significant. IFN- γ over-expression produced statistically significant positive outcomes while TNF down regulation lead to a worse survival. Three transcription factors, t-bet (TBX21), Signal Transducer and Activator of Transcription factor-1 and -4, Stat1 and Stat4, respectively regulate Th1 cell functions. T-bet and Stat-4 failed to show any

significance. Stat1 showed a poorer survival, but we can't eliminate the possibility that glioma cells themselves directly utilize stat1 [23]. Thus, the classic Th1 cells where all Th1 associated cytokines and transcription factors that are simultaneously made, were not really displayed within human clinical gliomas.

Th2 cells are classified by their production of the cytokines IL3, IL4, IL5, IL6, IL10, IL13, and IL25. Interleukins 3, 4, 10 and 13 failed to show any significant relationship with glioma survivorship. The over-expression of IL6 and IL25 lead to poor patient survival, while over-expression of IL5 forecast better survival of glioma patients. Looking at the transcription factors that control the fate of Th2 (Gata-3, Stat6, Stat5 and MAF) also displayed a mixed effect. Stat6 was not significant, while Stat5, and MAF correlated with poorer survival. Again the secretion profiles predicted by current dogma within *in situ* Th2 cells gliomas are not consistent with current experimental conclusions about Th2 cells and their global cytokine expression pattern.

A few not so well known T helper subsets (Th9, Th22 and Tfh cells) are easily studied by microarray analyses. As a result of exposure to antigen with IL4 and TGF- β , Th9 cells can be generated. Th9 can release IL9 and IL10. As described above for these cells, IL10 didn't have any relationship with glioma survival. IL9 had a mixed expression, both under-expression and over-expression both had poor prognoses. Hence Th9 cells probably do not play an important role in glioma immunology. Th22 cells are induced after antigen exposure when IL6 and TNF are also present. These cells produce IL13 and IL22. Both cytokines appeared to be devoid of any significance, so the contributions of Th22 are probably minor. Tfh (follicular helper) cells are stimulated by IL6, IL21 and IL27. Tfh produce IL6, IL10 and IL21. Both IL6 and IL21 lead towards poor survival. Thus, Tfh cells are suspicious and may contribute to the poor response of CD4+ cells in glioma patients.

Treg cells are currently in vogue explaining poor anti-tumor immunity in many tumors including gliomas [24]. TGF- β and IL2 drive the appearance of Treg. IL2 as mentioned earlier, trends towards a favorable patient survival, however the IL2R α (CD25) gene didn't show any significant relationship contradicting the current dogma that IL2R α + Treg are as predominant in GBM as previously thought [25]. The presence of TGF- β 1 and TGF- β 2 mRNA showed a very poor survival, glioma cells do make TGF- β 1 and TGF- β 2, so the initial source of stimulation of Treg may come directly from the glioma cells. Upon stimulation with TGF- β , the Treg can also release this cytokine or express the membrane form of TGF- β and thus amplifies the glioma's immunosuppressive nature. Treg use the FoxP3, Smad3 and Stat5 transcription factors. Stat5 and Smad3 showed a correlation with poor patient survival, but gliomas can also use smad3 and Stat5 [26,27], so the contribution of these transcription factors from Treg can't truly be assessed. FoxP3 is thought to be very specific for Treg, but it's presence within gliomas actually failed to show any significant correlation with patient survival. IL10 is released by Treg *in vitro*, but REMBRANDT doesn't show any survival relationship of IL10 production with gliomas, as described with Th2 and Th9 cells. The various mechanisms by which Treg can inhibit the immune system will be described in the next section (see Section 5: Immunosuppression). Thus, the previously held belief that Treg are solely responsible for poor patient performance via various immunotherapies, needs a critical reassessment.

Th17 cells type also uses TGF- β to help drive their initial activation. In addition, these Th17 precursor cells require IL6, IL21, IL1 α and IL1 β to become polarized into the Th17 phenotype after the initial T cell receptor engagements. IL6 and IL21 over-expression correlated with poor patient survival, while the expression of IL1 α or IL1 β subtypes didn't. Th17 cells have been identified in gliomas [28], but their precise role isn't defined. For melanomas, Th17 cells can mediate tumor destruction [29], but in colon cancer Th17 helps tumors grow [30]. REM-BRANDT analysis of the transcription factors used by Th17: RORC (ROR γ T), RORA (ROR α) and Stat3 did not show any positive or negative effects. Th17 cells release IL17A, IL17F, IL22, IL21 and IL26. The first three cytokines fail to correlate with patient survival. IL21 does show a poor patient survival prognosis, while IL26 shows a better patient survival. So the final verdict about Th17 cell's role in glioma is still undecided.

In conclusion, our current understandings of Th helper subsets seem to show multiple cytokines being expressed by cloned cells *in vitro*. However these distinct subsets can not be firmly identified *in situ*. This complexity is probably due to the complex cytokine/growth factor/environmental factors being produced within the tumor by the tumor, Treg or immune cells. One might even have to postulate subsets of these cells; e.g. Th1.1, Th1.2, Th2.1, Th2.2, Treg1.1, Treg 1.2, *etc* to explain these empirical observations. As described earlier, activated dendritic cell vaccines pulsed with autologous GBM tumors do improve GBM survival, so it will be important to describe what cytokines or subsets are correlating in those patients that are positively responding to such therapies.

CD8a is a more promiscuous marker, since it can be found on CTLs, NK, DC and other cell types. CD8b expression is exclusively found on CTLs; there were only 11 patients which over-expressed CD8b, while 89 patients down-regulated CD8b. The down-regulation of CD8b did however have a poor survival although not significant. Eomesodermin (Eomes) is a transcription factor reported to control CTL function [31]. There was trend towards lower Eomes expression with longer patient survival occurred in 11 patients. Twenty patients over-expressed this gene, but this profile didn't alter patient survival when compared to all glioma patients. Hence, the evidence for a beneficial role of CTLs *in situ* seems to be weak.

NK cells and $\gamma\delta$ type T cells are innate immunity-based lymphocytes. We examined a couple of NK/ $\gamma\delta$ related genes [32]: NKG2D (KLRK1) and NKp30 (NCR3). Both of these genes when they were down-regulated lead to poor survival. Conversely, when patients presented higher expression, this produced a better survival. Both findings had statistical significance. Thus, these innate type immune cells seem to show a dose-dependent *in vivo* effectiveness. This at first glance seemed unexpected. Two targets of $\gamma\delta$ type T cells are the MICA and MICB molecules [32]. Both of these molecules when over-expressed in glioma had poor prognoses, while low expressing gliomas had better outcomes. Hence the use of $\gamma\delta$ type T cells to treat gliomas might be a good clinical approach [33-35]. CTLs, NK and $\gamma\delta$ T cells use perforin (PRF1) and granzymes A and B (GRMA and GRMB) to actively lyse tumor targets *in vitro*. None of these gene expression patterns was associated with beneficial or deleterious effects. The exact mechanism by which NK/ $\gamma\delta$ type T cells could have anti-glioma tumor effects is still unknown. This anti-tumor immunity might not actually be mediated through direct cell-mediated

cytotoxicity using perforin and granzymes but could be through the release of unknown cytokines or other unknown effector molecules.

One thing that can be concluded from this REMBRANDT analysis dealing with current tumor immunity is the *in situ* process is much more complicated than was previously concluded from experimental *in vitro* conditions. Hence new tools need to be developed to explore the anti-tumor mechanisms that are present within the *in situ* tumor.

5. Immunosuppression

It has been known for awhile, that advanced cancer patients, including gliomas, have abnormal and diminished immune responses [36]. This impediment undoubtedly prevents the successful application of any cellular immunotherapeutic modality and helps explain the complex T cell immune responses described above. Over the years, it has postulated that a wide range of cytokines, growth factors and micro-environmental factors (hypoxia, reactive oxygen and nitrogen) lead to diminished immunological responses [15,37,38]. Figure 4 shows these potential causes of glioma/immune suppression pathways that have been postulated over the years. Besides immunosuppressive molecules such as transforming growth factor- β subtypes 1-3 (TGF- β subtypes 1-3), interleukin-10 (IL-10), vascular endothelial cell growth factor (VEGF) or prostaglandin E (PGE), there are also suppressor cells such as T regulatory cells (Treg) and myeloid derived suppressor cells (MDSC) that contributes to the overall immunosuppressive microenvironment.

REMBRANDT data analysis does show that some cytokines/growth factors have good statistical inverse correlations with patient survival. TGF- β 1 and TGF- β 2 inversely correlate with patient survival in a dose-dependent manner (high expression correlated with poor survival, while low expression prognosticated a better survival). TGF- β 3 doesn't appear to play much of a significant role within glioma patients. Interleukin-10 previously considered to play a major role in immunosuppression doesn't seem to have that much of an effect as seen above with the Th2 and Th9 phenotypes. VEGF inhibits immune activity via inhibitory actions upon dendritic cells [39]. Immune responses within the brain are initiated by the local dendritic cells [40]. Both VEGF-A and VEGF-B over-expression significantly correlated with poor patient survival, while VEGF-C and VEGF-D failed to show any deleterious behavior. Thus, VEGF-A and VEGF-B besides promoting angiogenesis of brain cancers promotes immune suppression.

Treg and MDSC suppress the immune system through different mechanisms. Treg can release TGF- β or express cell surface TGF- β (as described above). Treg can release indoleamine-oxidase-1 (Ido-1) in various tumor models [41]. Ido-1 helps degrade the amino acid, tryptophan. T cells are very sensitive to tryptophan deprivation and become inactivated by this condition. REMBRANDT shows a significant detrimental effect when IDO-1 is over-expressed, while in those patients showing reduced expression of IDO-1 a better survival was seen. In both cases, this data was significant. Human GBM cell lines don't seem to make Ido-1, so the contribution of Ido-1 is probably from the Treg. Cytotoxic T lymphocyte antigen-4 (CTLA-4), Programmed Cell Death-1 (PCD-1), Fas Ligand (FasL), B7-H1, B7-H3 molecules which are

membrane molecules and can inhibit other effector lymphocytes are possible immunosuppressive pathways of Treg. PCD1 over-expression was not associated with diminished survival, but under-expression did statistically show improved survival in a cohort of 104 patients. FasL and B7-H1 didn't show any survival effect, and are probably eliminated in their role in glioma immunology. B7-H3 was over-expressed in 203 patients and did inversely correlate with survival in a statistically significant way. There are currently thought to be two types of Treg, the natural and induced Treg subpopulations. When we examined the cell surface markers for either induced or natural Treg, nothing was immediately obvious. We postulate that *in situ* glioma Tregs are Foxp3-, FasL-, TGF-β+, Ido1+, PCD1+ and B7-H3+ based upon evaluation by REMBRANDT.

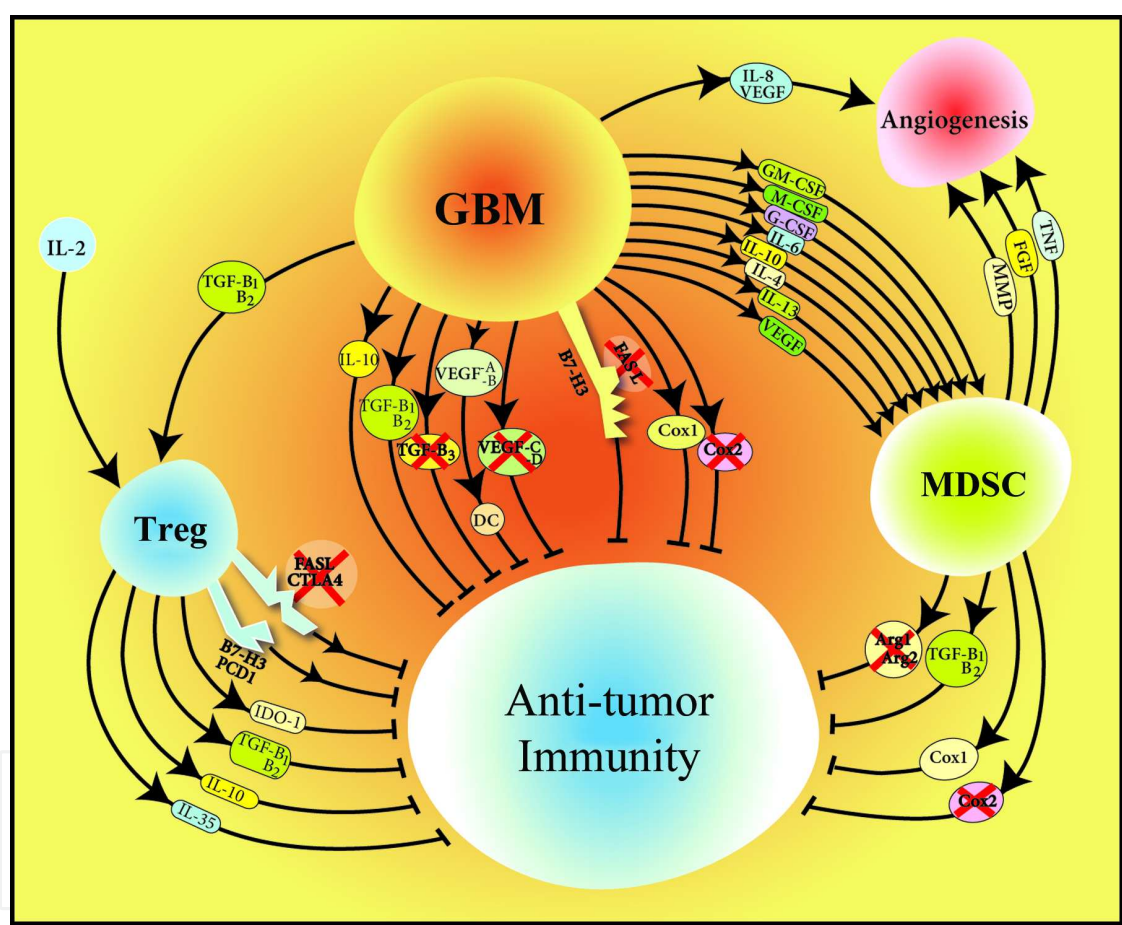


Figure 4. Mechanisms of immunosuppression found within gliomas. As a result of the glioma cell release growth factors and cytokines T regulatory (Treg) and myeloid derived suppressor cells (MDSC) are recruited. Glioma cells can express membrane proteins Fas Ligand and B7-H3. For sake of simplicity only 1 transmembrane protein is shown. Likewise Treg can express transmembrane inhibitory molecules FasL, CTLA4, B7-H3 and PCD1. A Red X indicates the pathways by REMBRANDT analysis are not likely playing a major role pathways. MDSC and gliomas can release Cox 1.

MDSC can either be composed of CD33+ cells or CD14+ cells [42]. These markers can be found on either the granulocytic or monocytic types of MDSC. MDSC are stimulated by a wide variety of cytokines and growth factors found in gliomas: GM-CSF, M-CSF, VEGF, IL1, IL4, IL6, IL13 and PGE, most of these cytokines were over-expressed by gliomas as determined by REM-

BRANDT. Fifty glioma patients did demonstrate an earlier demise when CD33 was determined to be over-expressed. In contrast only 8 patients down-regulated expression of CD33; this observation was not considered significant. CD14 expression was increased in 186 patients and possessed significantly shortened lives. Seventeen glioma patients were CD14 down-regulated and were approaching a significant survival advantage ($p=0.058$). So both types of MDSC can reside within human gliomas. MDSC can release TGF- β , arginase or prostaglandin (PGE) that prevents T cell expansion through different pathways [42]. As described above TGF- β 1 and TGF- β 2 are inversely correlated with glioma survival. But TGF- β can come from either Treg, the glioma itself or from MDSC. Arginase suppresses T cell function in a manner similar to IDO, in that T cells also require sufficient arginine for proper function. Two isoforms of arginase are known, Arg-1 and Arg-2. Arg-1 is a cytoplasmic enzyme; Arg-2 is a mitochondria contained enzyme. Neither form of arginase had any significant effect, eliminating this likely pathway from occurring in human gliomas. Thus, the way that MDSC mediate their actions is probably through TGF- β or PGE.

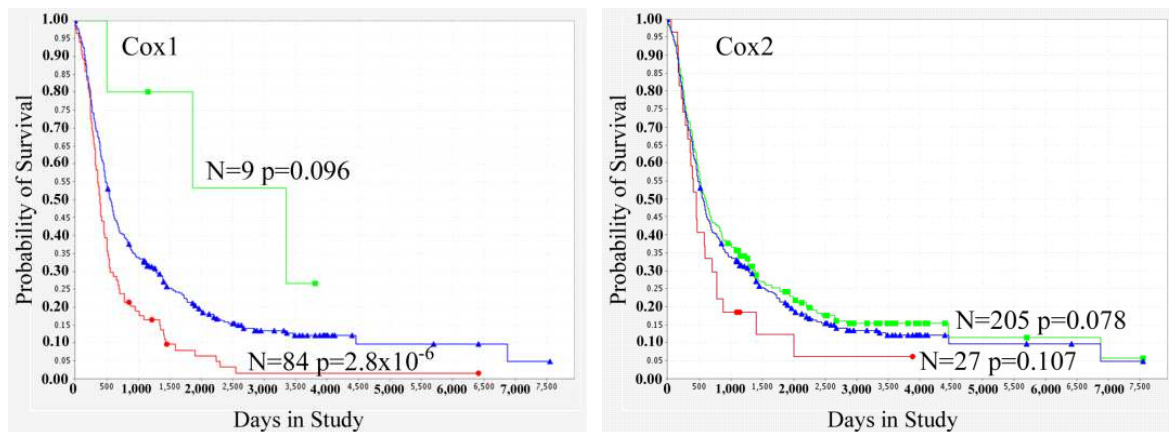


Figure 5. Survival statistics of glioma patients with Cox 1 and Cox 2 differential gene expression using REMBRANDT. The Left Panel shows the survival statistics of glioma patient analyzed for Cox 1 expression. There were 9 patient under-expressing Cox 1, which failed to achieve statistical significance. Eighty-four patients over-expressed Cox 1, which did reach a significant p value. The Right panel shows the REMBRANDT analysis of Cox 2 expression. There were 205 patients that under-expressed Cox 2, while only 27 patients over-expressed the Cox 2 transcripts. Neither phenotypes were considered significantly different from the total population of gliomas.

PGE₂ is synthesized by either the cyclo-oxygenase-1 (Cox-1) or -2 (Cox-2) enzymes [43]. Cox 1 is considered by some to be a house-keeping gene and is constitutively produced and synthesizes low amounts of PGE₂. Figure 5 shows the REMBRANDT data dealing with the cyclo-oxygenases. Cox-2 is inducible and produces much more PGE₂ when cells are properly activated. Many tumors [43], including glioma cells [44,45] are reported to over-express the Cox2 enzyme. Cox2 derived epitopes have been described in human esophageal cancers that CTLs can recognize [46], so this enzyme might be a potential human glioma antigen, too. Epidemiological studies have shown that a daily dose of aspirin helps reduce PGE₂ production and lowers the incidence of a variety of cancers [47]. When Cox2 was analyzed by REMBRANDT, there was an association showing that gliomas that over-expressed Cox2 (27

patients) showed an unfavorable survival, while the under-expression of Cox 2 in 205 patients had only slightly improved patient survival. Neither of these expression patterns showed statistical significance when compared to all gliomas. Cox2 production of PGE might not therefore be a major source of PGE production as previously thought. When Cox1 expression was examined as a control, a surprisingly better correlation was found with patient survival. There were only 5 patients who under-expressed Cox 1, so statistically, this is not considered significant. Eighty-four glioma patients who over-expressed Cox1 succumbed earlier to their cancer. This later group of patients did achieve a very good statistical significance ($p = 2.8 \times 10^{-6}$).

PGE₂ is a vasodilator and it is involved in angiogenesis so it may have multiple roles in glioma biology, besides immune suppression. Only prostaglandin receptors EP2 and EP4 were elevated within human gliomas using REMBRANDT. Based upon this REMBRANDT assessment, the role of Cox-1 needs to be investigated further in glioma biology and could be a target for therapy in some patients.

6. Growth factors, cytokines, chemokine are there targets of immunotherapy by T cells

PGE₂ receptors are coupled to G-proteins that activate adenylate cyclase, leading to an increase of intracellular cAMP which activates protein kinase A (PKA). This cellular signaling results in increased production of Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF) that assists new blood vessel formation (angiogenesis) (Figure 6). Glioma cells also possess growth factor and cytokine receptors, such as the interleukin-13R α 2 (IL-13R α 2) [48], EphA2 [49], platelet derived growth factor receptor (PDGFR2) [50,51], epidermal growth factor receptor (EGFR)[52], insulin-like growth factor receptor (IGFR)[53], c-met (which binds hepatocyte growth factor/ scatter factor (HGF/SF)[54], interleukin-6 receptors (IL-6R) [55], and GM-CSF receptors [56]. Table 2 summarizes this data of growth factors/cytokines expressed within human gliomas along with their respective receptors. It can be speculated that some glioma cells use these potential autocrine growth pathways. REMBRANDT does seem to indicate that PDGF-B and PDGFR2 and the IL6/IL6R pathways are viable models of this autocrine process. The other cytokine/growth factors could be indicative of paracrine routes. Upon proper binding to their receptors, most growth factors/cytokines (EGF, IGF, PDGF, FGF, HGF/SF, Ephrin A, IL-6, IL-13 and others) use the PI3 kinase family of signal transducing docking molecules [57,58].

Upon ligation of the receptor, the Rac, Ras and Raf1 docking proteins are interacting and activate PI3K. H-ras and N-Ras have a positive correlation with poor glioma survival. Members of the Ras family, rab4,5,8,11,21,25, have been investigated with multiple aspects of signal transduction with glioma cells. But REMBRANDT showed that these rabs didn't have any significance. PI3 kinase family members do have a strong linkage with poor glioma survival by our analysis of the current data. Akt1 or Akt2 is also activated and does also show the same inverse relationship with patient survival. Thus, targeting PI3K/Akt dependent pathways are still a valid target.

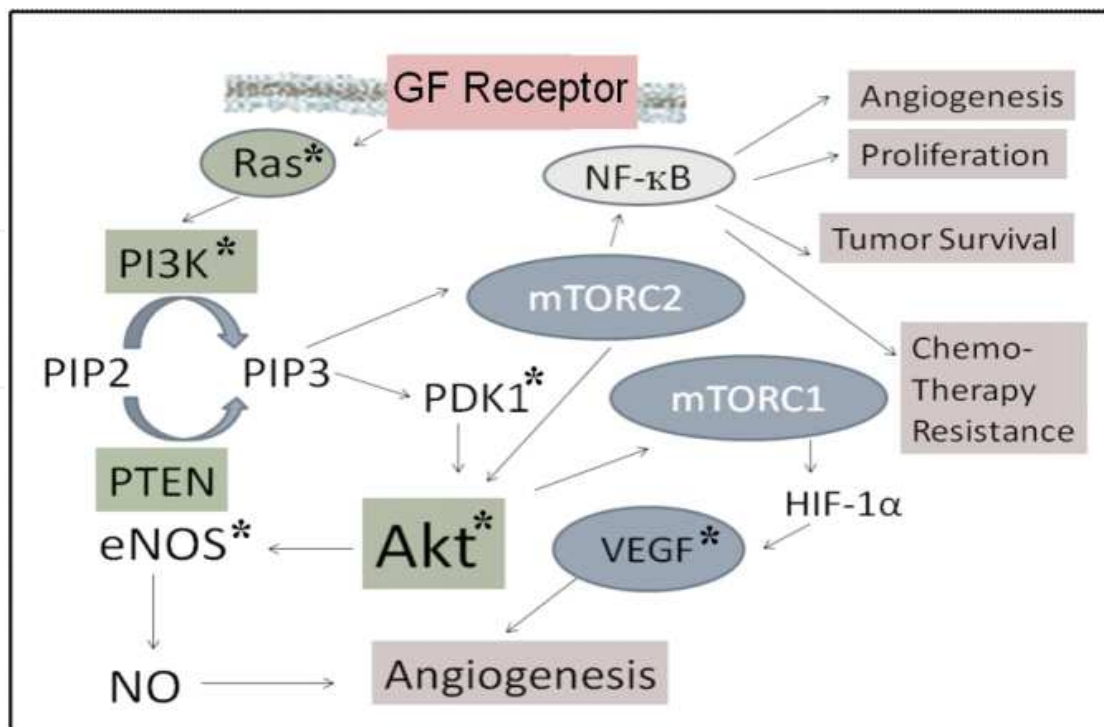


Figure 6. Generalized mechanism by which growth factor/cytokine interactions can initiate down-stream biochemical pathways. Upon binding a growth factor or cytokines, Ras/Raf/Rac signal transduction begins. PI3 kinase converts PIP2 into PIP3. PIP3 stimulates PDK1 which in turn activates Akt. eNOS is turned on by Akt. The asterisks indicate that protein is over-expressed and leads to poor patient survival by REMBRANDT.

The normal brain contains a variety of neurons, Schwann cells, glial cells, microglia and other cells. These cells are held in place and are embedded by an extracellular matrix (ECM)[59,60]. Matrix proteins besides providing cell anchorage, can also bind and sequester various soluble biological modulators. Table 3 also lists the various extracellular matrix proteins found within the brain that can bind to various glioma-related cytokines/growth factors. Several growth factors upon binding to their extracellular matrix enhance binding affinity to their receptors. Since the soluble factor is tethered to a large matrix protein, endocytosis may be slowed and allowed for increased intracellular signaling to be mediated. A prolonged signal transduction via PIP₃ can also recruits intracellular cytoskeletal elements Wave/WASP-like proteins which allows actin polymerization to be initiated at this membrane site, this can then help explain the presence of filopodia, lamellopodia, microvilli and invadopodia of gliomas [61-64]. See Section 7.2.

7. Mechanisms of invasion of gliomas

7.1. Extracellular mechanisms

It has been concluded that the reason why many human gliomas cannot be cured by conventional surgical, chemotherapeutic and radiation modalities is these tumors are very in-

filtrative. After the neurosurgeons and the radiation oncologists treat their patients, some remaining cancer cells have escaped these interventions. Perhaps the glioma cancer initiating cells or “stem cells” have even already migrated into new areas of the brain in searching for new sources of cytokines or other stimuli (see Section 6). There are more components that enable the glioma cells to become more invasive and can become potential targets of some therapy.

Glioma cells possess receptors/molecules than can bind to environmental components. These receptors can be either cell-adhesion integrins or CD44 [65]. The most important integrins are considered to be the $\alpha_v\beta_3$, and $\alpha_v\beta_5$ heterodimers [66]. These integrins act as receptors to bind vitronectin, fibronectin, osteopontin and cyr61 [67]. The last three ECM proteins when they were over-expressed also showed a predicted poor survival with glioma patients. Two other integrins, $\alpha_6\beta_1$ [68] and $\alpha_5\beta_1$ [69], have been reported to also play roles in glioma tumorigenesis. The $\alpha_v\beta_3$ and $\alpha_6\beta_1$ integrins are functional receptors for several heparin binding growth factors, pleiotrophin and midkine, respectively [70,71]. Midkine has been identified as a potential mediator that promotes chemoresistance within those midkine-stimulated cells [71]. As described in Table 3 some of these genes that interacted with the cytokines and growth factors were identified by REMBRANDT as significant in predicting patient outcome. REMBRANDT confirmed that CD44, α_v , α_6 and midkine lead to poorer prognoses.

Cancer cells, including gliomas do release matrix metalloproteinases (MMP) which digest the surrounding tissue and extracellular matrix allowing the cancer cells to invade surrounding tissue. By REMBRANDT we identified that MMP1, MMP2, MMP9, MMP11 and MMP14 all were associated with a bad prognosis. A natural inhibitor of the MMP, called TIMP3, associated with better patient survival when over-expressed, as current dogma predicts. So strategies that counter these MMPs or by increasing TIMP3 activity might have an important role in preventing the glioma cells from breaking its initial containment.

Gliomas might also release excess glutamate and kill surrounding normal tissue via an excitotoxic process [72]. There is speculation that gliomas can also down-regulate their glutamate receptors [73,74] so that they become impervious to the cytotoxic levels of glutamate that they release. Glutamate can be synthesized from the enzyme glutamate synthetase (GLUL) or can be scavenged via numerous catabolic pathways. Glutamate can be released via exporters that use K^+ and Na^+ cation exchangers, or SoLute Carriers (see Section 7.3 below). We found that there was no correlation with GLUL expression with patient survival or demise. So the most likely source of the released glutamate is coming via protein catabolism. The glutamate receptors GRIA1, GRIA2, GRIA3, GRIA4, GRM5 and GRM7 all correlated with a bad prognosis by a REMBRANDT analysis when they were down-regulated. The interpretation here is consistent with the initial hypothesis that this down-regulation by the glioma cells can make themselves resistant to the cytotoxic levels of glutamate found in the local microenvironment.

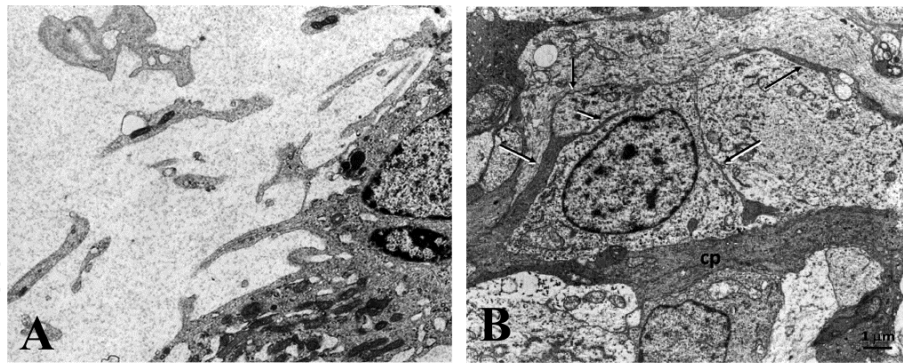


Figure 7. Electron microscopy of human GBM *in situ*. Panel A shows gliomas with microvilli invading surrounding extracellular matrix: magnified 15,000 x times. Panel B shows gliomas with filopodia invading through other cells. Magnification 5,000 x times.

7.2. Microvilli and filopodia

Cell surface superstructures including microvilli, invadopodia and filopodia have been described over the years [75-79]. These structures allow tumor cells to probe for weak spots in surrounding normal cells and then begin to invade that area as described above. Some of the gliomas found in GBM do have these microvilli structures *in situ*. Some microvilli actually had mitochondria within them (Figure 7A), and their apparent function appears linked with the ROS generation and subsequent activation of several pathways essentials for glioma invasiveness [80]. Thus, microvilli have a built in power supply to immediately supply the energy into their probing mechanisms via re-organization of microfilaments and microtubules. At some edges there were long filopodial projections that could extend past two cell- lengths. Some glioma cells possess these long filopodia and thus have the ability to probe into distant sites (Figure 7B). Glioma cells like U251 display her2/neu on their microvilli and filopodia [81]. We have also seen the receptors such as: IL13Ra2, EphA2, EGFR, PDGFR, c-Met, IGFR and IL6R on the microvilli and filopodia of U251 gliomas [unpublished data]. These microvilli also provide a novel “sea urchin” type defense that prevents various lymphocytes from mediating cytotoxicity. Thus these microvilli and filopodia can be used for multiple purposes by glioma cells and assist in their invasiveness.

We searched out the molecules that have been associated with microvilli, lamellipodia and filopodia with REMBRANT to identify pathways that are most likely being used by human gliomas. Figure 8 shows a basic schematic of how these superstructures could be composed of actin-related proteins. Microfilaments, actin-binding proteins and microtubules have been reviewed in [82-84]. Microfilaments are composed of the actin family members. There are two types of actin. The monomeric, globular (G-form) represents the single unit, whereas, when these monomers polymerize, they form the filamentous, F-actin type. Actin contains both pointed and a barbed ends. The barbed end is protruding from the growing end of the F-actin polymer. Actin plays many roles in cellular processes. Actin anchors many membrane-bound proteins in place and are associated with glioma cell motility and the cytoske-

leton. Surprisingly, by REMBRANDT, there was no significance of actin expression. So glioma cells probably just recycle and reuse their actin in a more efficient manner.

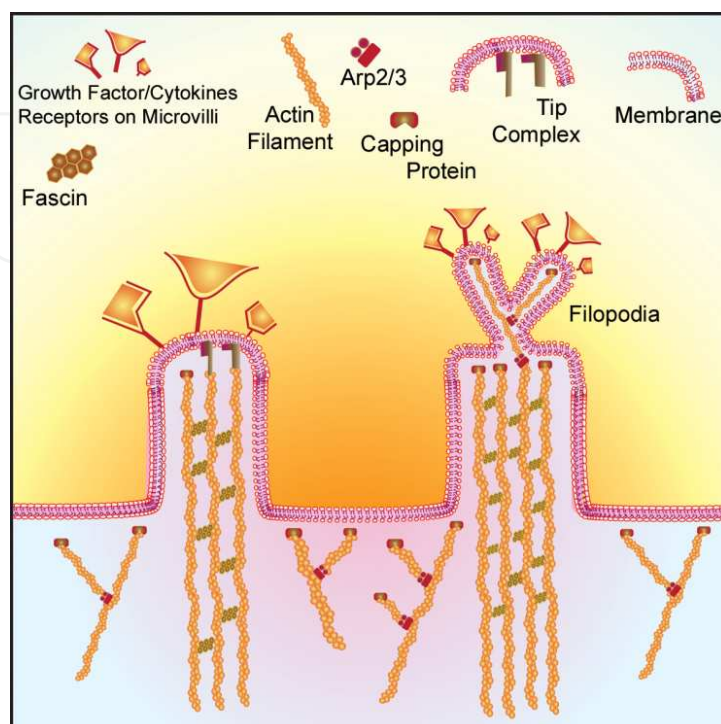


Figure 8. Schematic diagram of microvilli/filopodia. The microvilli (left) and a microvilli with a branched filopodia (right) are shown. Various growth factor/cytokine receptors and cell adhesion molecules are found on these structures. Upon binding with their ligands, the transmembrane receptors become activated and allow the nucleation of various cytoskeletal elements to begin. Hence the activated receptors can now begin a process where cell polarity is initiated.

There are molecules that crosslink the actin locking proteins together. Some proteins bundle actin polymers together making these proteins stronger, fascin is a prime example. Actin-binding proteins like Arp2/3 cause branch points within the polymerized actin strands and help explain the bending of the filopodia [85]. At least 100 proteins can associate directly or indirectly with actin some of the more important ones are listed in Table 3. It is therefore not surprising that actin-binding proteins are associated with the mobility of many cancer cells. The proteins, fascin, podoplanin, and myosin IIB, have been actively studied with regards to cancer cell motility [86-90]. These two proteins are also over-expressed in gliomas and in other cancers. The over-expression of fascin and podoplanin mRNA within gliomas is linked with a worse prognosis in those patients. Actin and fascin are important for maintaining the lamellopodia, microvilli and filopodia of various cells (Figure 8). This infiltrative nature of gliomas is believed to be the prime reason why this tumor remains such a lethal cancer, despite the best efforts to surgically remove and irradiate the surgical incision sites. So these two molecules can be considered good targets for possible molecular intervention. Table 3 summarizes as many cytoskeletal elements which have been associated with cell invasion and migration over the years and correlated them with significance by REMBRANDT.

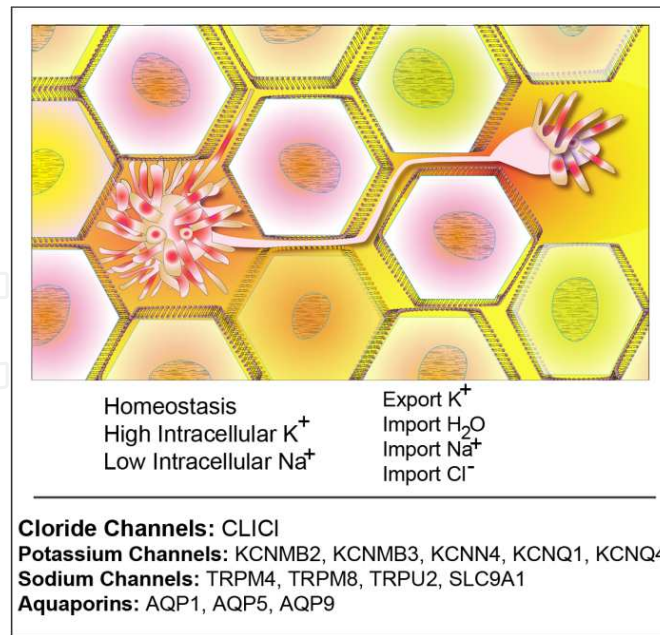


Figure 9. Proposed model of ion channel regulation of glioma cell invasion. The main cell body (left) is in normal ionic homeostasis, where there are high intracellular K^+ ion concentrations with relatively low concentrations of Na^+ ions. The invading part of the cell (right) is now swelling as a result of possible activation of several ion channels listed at the bottom. K^+ ions are exported while Na^+ and Cl^- ions along with water is imported causing the cell swell. As a result of the swelling at the leading edge the rest of the main cell body can be pulled into the new area. The bottom lines list the possible ion channels/aquaporins that were identified to be of significance by REMBRANDT.

7.3. A mechanism for glioma cells to squeeze through normal tissue

Harald Sontheimer has pioneered ion channel discovery in brain cancers. His unified hypothesis is that gliomas can shrink at the leading edge of the glioma cell [91]. In this way, the glioma cell then behaves like an amoeba and pushes itself through the normal tissue. Figure 9 shows a simplistic model of the dynamic aspects of ion-driven movements in a slightly different model 19. While at rest and in homeostasis, the glioma cell maintains its normal physiological ionic composition and has multiple filipodia projections. Some projections find an area which stimulates proper intracellular interactions and begin an invasive process. Here there is high intracellular levels of K^+ , and normal concentrations of Na^+ , Ca^{+2} , Cl^- and water. At the leading edge of the cell, now a disruption of normal ionic homeostasis occurs. Here K^+ channels are activated and K^+ ions are released, while Na^+ ions are allowed inside. As Na^+ ions enter, so does Cl^- anions and water, hence the cell can expand within this region of the cell. Colman et al., [92] found that one of the channels that are strongly correlated with poor survival was a CLIC1 chloride channel. REMBRANDT confirmed this ion channel expression inversely associated with poor patient survival. This chloride channel, CLIC1, also can share a subunit ($BK\beta$ as known as KCNMB2 or KCNMB3) with the big potassium ($BK\alpha$, KCNMA1) ion channel channel [93]. REMBRANDT analysis showed the $BK\beta$ 2 or $BK\beta$ 3 chains had an inverse correlation with survival, like the CLIC1 expression. The KCNMA1/ $BK\alpha$ ion channel failed to show any relationship with glioma patient survival. This last finding prompted us to consider whether other potassium channels could provide an alternative pathway for exporting of K^+ ions instead. The

potassium channels are associated with poor glioma survival are KCNN4, KCNQ1, and KCNQ4.

For cells to maintain electroneutrality, as K^+ ions are eliminated Na^+ ions must enter. REMBRANDT analysis reveals that four sodium ion channels are strongly correlated with an adverse survival rate. These sodium ion channels are TRPM8, TRPM4, TRPV2 and a sodium-hydrogen exchanger (a.k.a. NHE1, SLC9A1). As sodium ions enter the cell, so does water, so the aquaporins (water channels) are playing an active role in this dynamic swelling process, too. The aquaporins, AQP1, AQP5 and AQP9 all show dose-dependent inverse pattern of survival within glioma patients. Interestingly, AQP7 and AQP10 display an opposite pattern than the previous three aquaporins. Here over-expression of AQP7 and AQP10 lead towards better survival, while lowered expression produced a worse prognosis.

Sontheimer's lab found that a novel spliced version of the BK channel, called the glioma BK channel is found in human gliomas [94]. We created an antibody directed towards this region and confirmed that all human gliomas and many rat and mouse glioma cells lines also possessed gBK [95]. We also found that two epitopes within these gBK regions can induce human HLA-A2 restricted CTLs which lyse a variety of human cancer cells including glioma cells. Thus, ion channels have the potential to be developed into immunotherapeutic targets. So the ion channels, CLIC1, KCNMB2, KCNMB3, KCNN4, KCNQ1, KCNQ4, TRPM4, TRPM8, TRPV2 and SLC9A1 could also theoretically be targeted by immunotherapy. Dutoit and colleagues [96] have mapped a HLA-A2 derived peptidome which could stimulate possible immunotargets for CTL therapy. Some of these identified peptides did include ion and transporter channels, such as: ATP1A2, ATP2B1, CACNA1A, GRIA2, GRIA3 and SLC4A4. So immunotherapy might be directed towards these ion channels, aquaporins and transporters could be playing a role in glioma invasiveness.

7.4. "Death signatures"

Glinsky, *et al.*, [97] analyzed a wide variety of cancers by using microarray technology and discovered that a series of 11 genes (GBX2, Ki67, CCNB1, BUB1, KNTC2, USP22, HCFC1, RNF2, ANK3, FGFR2, and CES1) had a unique pattern. When this profile was expressed in those various cancers it had a high statistical probability that the patient will die of the cancer. They called this expression pattern, a "Death Signature". In theory, one would think that if an immunological intervention could be developed, these gene products might be perfect targets for immunotherapy, assuming that these genes or their synthesized proteins are actively playing a role in the cancer's aggressiveness which leads to the patient's demise. Since these genes foretold the patient death, if you could then vaccinate a patient against these antigens and prevent these genes from manifesting themselves, you should then interfere with their final outcome, death. When we examined the REMBRANDT survival data with this series of genes found in the Death Signature, there was actually very little significance; most of the gene expression patterns, when investigated individually were the opposite of what was predicted by the Death Signature concept. The only gene that correlated well with poor glioma survival was a marker for cell proliferation that pathologists regularly use as a sign of cell division, Ki67. Thus, we feel that this "Death Signature" doesn't fit too well with gliomas. In fairness to Glinsky and coworkers, they did use a large variety of cancers and they did do a multigene

analysis, which REMBRANDT currently does not allow. So any multifactoral effect may have simply been masked by the complexity of their analysis.

8. Targeting the genes commonly found in human GBM

In 2010, Colman and colleagues reported a series of 31 genes that were associated with poor GBM survival [92]. When Rembrandt analysis was done using these genes, the vast majority of these genes correlated extremely well. This evaluation was much better than “Death Signature profile”. Since the MD Anderson and University of California, San Francisco groups include many of their patients into REMBRANDT, it would be expected a much better correlation could be found.

One interesting finding was that a couple of these genes from the Colman, *et al.* study were also found in the glioblastoma peptidome derived from HLA-A2 alleles obtained from GBM surgical specimens [96]. These T cells-derived antigens included: CHI3L1 (YKL-40), IGFBP3 (insulin-like growth factor binding protein-3), PDPN (podoplanin) and TNC (tenascin C). We further examined these 31 genes from the Colman study and found their protein sequences. We subjected them to SYPEITHI data analysis program (<http://www.syfpeithi.de/>) to determine the possibility of generating nonamer peptide determinants towards their potential antigenic peptides. Table 4 summarizes this study. We limited this inquiry to those peptides that are found restricted to the HLA-0201 allele, since that is the most common HLA allele found in the US with just a little less than half the Caucasians possessing this phenotype. Some of the Colman and colleague’s proteins are quite small ranging from 116 amino acids (dynein light chain) to fibronectin isoform1 that possess 2477 amino acids. For the dynein light chain there were only 12 predicted peptides that could bind to HLA-A0201 with a binding score of 16 or better. In contrast, the fibronectin isoform-1 had 236 possible peptides. In sum, these proteins provides 1,802 peptides that could theoretically be used to vaccinate patients against HLA-0201+ tumor cells. Hence this might provide a particularly rich source of very clinically relevant antigens to target glioma.

9. New targets

As we have described in Table 1, we only have 17 very good or good tumor antigens by which we can target brain cancers, thus new antigens are needed. REMBRANDT has validated *in silico* that there might be new targets that haven’t been considered before. It will be important to consider targeting these molecules using a variety of mechanisms since they cause poor patient survival and their under-expression leads to better survival. This can include immunotherapy, molecular gene silencing or even knock-in strategies to take advantage of those genes which prolonged patient survival. In Table 2 we show that several growth factors, their cytokines or their extracellular matrix proteins might be potential good targets. Table 3 illustrated many intracellular cytoskeletal elements that are also good targets for either

immunotherapy or potential gene knock-in. Colman's study [92] (Table 4) provided an incredible wealth of potential tumor antigens that could conceivably be targeted.

Growth factor/cytokine	Receptor	Extracellular protein capable of immobilizing
Acidic Fibroblast growth factor	FGFR*	glypican-1*
Basic Fibroblast growth factor	FGFR*	neurocan, syndecan-1*, -2*, -3, glypican-1*, -2, -3, phosphacan, nerve-glial antigen-2 (NG2) also known as chondroitin sulfate proteoglycan 4, phosphacan (PTPRZ1).
Unknown ligand	erbB2/her2*	not known
Transforming growth factor- $\beta 1^{**}\beta 2^{**}\beta 3$	TGFBR1* TGFBR2** TGFBR3	syndecan-1*, decorin**, dermatan sulfate
Insulin-like growth factor-1	IGF1R	glypican-3
Hepatocyte growth factor/	c-Met*	dermatan sulfate/chondroitin sulfate B
VEGF-A**, VEGF-B**	VEGFR1 (flt1) VEGFR2 (kdr)*	glypican-1*, syndecan-2*
Platelet derived growth factor-A	PDGFR1	NG2
Platelet derived growth factor-B*	PDGFR2*	
Pleiotrophin*	nucleolin, $\alpha_v\beta_3$ $A_6\beta_1$	syndecan-3
Midkine**	nucleolin, $\alpha_v\beta_3$ $A_6\beta_1$	various heparans
Neuroregulin-1	erbB3 erbB4	heparan sulfated proteoglycans
Fibroblast growth factor-4	FGFR1	heparan sulfate
SDF-1 (CXCL12)	CXCR4*	syndecan-4
Epidermal growth factor*	EGFR	syndecan-2*, laminin A2**, lamininB1**, laminin A5*, laminin B3*
Interleukin-6*	IL6R*	not reported yet
Interleukin-8*	IL8RA, IL8RB*	syndecan-2*
Interleukin-13	IL13Ra1** IL13Ra2**	not reported as yet.
Granulocyte-macrophage	CSF2R	syndecan-2*
Colony Stimulating Factor	c-fms	not reported as yet.

Table 2. Growth factors, cytokines and their receptors along with brain derived ECM. Single asterisk indicates either over-expression leads to poor survival or under-expression leads towards better survival. Double asterisk indicates both conditions described immediately above were true.

Thus, targeting some of these genes using DC based vaccines could encompass using whole tumor cell lysates, recombinant proteins or peptides using this information might be able to target these key proteins. One can also interfere with these vital functions of glioma invasion via molecular methods. In support of this concept, Dutroit and colleagues [96] using the peptidomics approach with HLA-A2 associated peptides has found evidence that some of these REMBRANDT validated peptides are present and could be possible targets of immuno-therapy if one was to use the same starting material that they used.

Signal transduction		
Good Potential antigen	No relevance	Opposite expression
CDC42, grb2, h-ras, n-ras	c-Src, Rac1, Raf1, rab4, 5, 8, 11, 21, 25	APC, k-ras
Actin Cytoskeletal		
Actn, Arf6, basigin, caveolin1, 2, CDC42, Crkl, diaph1, erm/ moesin, filamin A, C, fascin1, grb2, gelsolin, IQGAP1, 2, 3, Mrlc2, Myh9, Nck1, paxilin, Pdpn, Ppir12a, Pfn1, Rhamm, supervillin, vimentin, Was/nWasp, Wasf2	Abi2, Actr3, Arp2, Arp3, Bcar1, caveolin 3, cofilin, c-Src, Cyfip1, diaph3, dynamin1, 2, ezrin, f-actin, fascin2, GFAP, Lmk1, Mena/Enah, MLCK, Myh2, netrin, Pak1, 3, 4, profilin 2, RhoA, Sh3pxd2a/Tks5, Ssh1, vinculin, Wasl, Wipf1	Apc, Aref/ARHGEF4, ARHGEF7, doublecortin, dynamin3, dock1, Dtnb, FAK, fascin3, Myh10, Ntn4, Pak7, Stathmin 2,3, 4, Wasf1
Tubulin cytoskeleton		
Kif2c, Kif11/eg5	Dnch2, Dynll2, Dync1l11, Dynein light chain/tctex, Kif1c, Klc2, Tubb3,5, Tubg1,2	Kif3c, /kif21b
Membrane Protein		
CD44, Itga6, Itgav, Itgb1, Itgb5, Pdpn,	Itgb3	

Table 3. Cytoskeletal proteins associated with cell migration, invasion and structure analyzed by REMBRANDT. (+) indicates significant values, opposite (+) indicates a significant value, but is opposite to what is actually predicted by current dogma.

REMBRANDT was then used to determine whether this gene is either over-expressed with a bad prognosis or under-expressed with a better survival was. When either one of those conditions was met then that antigen was considered a potentially good antigen. If both conditions were met, then it is considered a potentially very good antigen. Conversely, if neither condition was met then it was considered not good. Opposite indicates the REMBRANDT analysis contradicts the expected prediction, where over expression lead to a better survival and vice versa.

The numbers of amino acids of the translated protein are shown. This protein was then analyzed by SFYPEITHI analysis looking at possible nonamers that can theoretically bind to HLA-A*0201, as a metric for how tentatively immunogenic this protein could be. SFYPEITHI scores better than 16 were then counted. REMBRANDT was then used to determine whether

Gene	# Amino Acids	Predicted # Epitopes	Rembrandt Correl
α-actinin-1 isoform a	914	122	Very Good
Aquaporin-1, isoform1	269	66	Very Good
Chitinase-3like protein 1	383	40	Very Good
Chloride intracell. channel protein 1	241	39	Good
Collagen α2	1366	95	Very Good
Epithelial membrane protein-3	163	45	Very Good
Fatty acid binding protein-5	135	14	Very Good
Fibronectin isoform 1	2477	236	Good
Transmembrane glycoprotein NMB	572	77	Very Good
Insulin-like growth factor bind. protein-2	328	41	Very Good
Insulin-like growth factor bind. protein-3	297	33	Very Good
Lactate dehydrogenase A	332	62	Very Good
Galectin-1	135	10	Very Good
Galectin-3	250	23	Very Good
Gycoprotein (transmembrane) NMB	572	44	Very Good
Monoamine oxidase B	520	84	Very Good
Nicotinamide N-methyltransferase	264	38	Very Good
Podoplanin a	238	46	Very Good
Proteolipid protein 2	152	34	Very Good
Transmembrane protein-158 (RIS)	300	50	Very Good
Serpine A3	423	80	Very Good
Plasminogen activator inhibitor 1	402	66	Very Good
Plasma protease C1 inhibitor	500	92	Very Good
Protein S100-A10	97	15	Good
Transgelin	201	21	Very Good
Transgelin 2	199	24	Very Good
Dynein light chain (Tctex)	116	12	No Good
Transforming growth factor-β1	390	57	Very Good
Tissue inhib. Metalloprot inhibitor 1	207	31	Very Good
Thymosin β10 (TMSB10)	44	0	Very Good
Tenascin	2201	179	No Good
VEGF-A	412	28	Very Good
Total Potential T cell epitopes: 1,802			

Table 4. SYPEITHI predicted binding epitopes for the genes restricted to HLA-A*0201 that were over-expressed in glioma and correlated with poor survival.

this gene is either over-expressed with a bad prognosis or under-expressed with a better survival. When either one of those conditions was met then that antigen was considered a potentially good antigen. If both conditions were met, then it is considered a potentially very good antigen. Conversely, if neither condition was met then it was considered not good.

10. Limitations and improvements for REMBRANDT

REMBRANDT type databases will be the future analyzing other types of cancer. Overall, this program is quite useful and easy to use. One must have the proper abbreviation of the gene, which can usually be found at websites like Gene Cards, Wiki Genes or the HUGO Gene Nomenclature Committee HGNC databases. Occasionally for some of the genes we searched, our analyses ran into some technical problems that won't perform the computations, obviously some glitches remain in the system that will be eventually ironed out. For the next version of REMBRANDT it will be important to allow the glioma sub-classes to be assessed: proneural, mesenchymal and classic GBM. This option will be important, since each GBM subclass has its own particular targets to be used; such as growth factor requirements proneural/neural GBM use the PDGF pathway, while classical GBM use more EGF driven pathways. YKL-40 is a marker of the mesenchymal GBM subtype and it might be a way to identify key interactions. Multivariant gene analysis, where one can analyze several genes simultaneously will also allow better analyses. So REMBRANDT version 2.0 should be enhanced with this kind of analysis.

The data we analyzed was based on gene expression measuring mRNA levels using microarrays. So the danger is that these conclusions does not necessarily mean that the corresponding proteins were synthesized. Splice variants are also not taken into account. For example gBK and BK α channels cannot be distinguished as separate isoform resulted from the current microarray platform. Nevertheless, this type of analysis will provide neuro-oncologists with some exciting concepts to think about for years to come.

11. Conclusions

After many frustrating years of attempting to use immunotherapy against cancers, tumor immunologists are starting to see successful results in melanomas, prostate cancer and in some GBM patients including high grade gliomas. The identification of better glioma-associated antigens is paramount to continue this investigative process. Many defined antigens have been discovered by serendipity and this random process has lead to developments such as using a variety of modalities: immunotoxins, chimeric antigen re-directed T cells, molecular silencing approaches, DC-based vaccines, *etc.* But there are undoubtedly many more antigens still to be discovered. Microarray analysis of tumors has already been proven to be a powerful way of classifying tumors and provide novel tumor-specific therapies. The REMBRANDT data portal allows researchers a chance to quickly assess this microarray data and quickly test potential therapies *in silico* as we have attempted to do here before doing expensive clinical trials. REMBRANDT gives one a chance to determine whether a gene has any importance in glioma biology using a large data base. In this chapter we showed that REMBRANDT offers oncologists a chance to analyze various biological pathways to see if predicted molecules seen in tissue culture cell lines are applicable with the *in situ* scenario. REMBRANDT offers immunologists a chance to now make use of the key genes involved in gliomas and then target them

for immunotherapy. By targeting critical genes by therapy, one hopes to attack cancers at their strong points. REMBRANDT is pioneering this ability and its ability will undoubtedly will be copied by other medical oncologists in studying their cancer of interest. So this is the first step towards using knowledge to specifically target cancer.

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