Alternative RNA Splicing in the Pathogenesis of GBM

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Abstract—Alternative splicing enables the generation of different proteins from a single gene, greatly increasing the use of genetic information. The resultant protein isoforms often have different biological properties effecting the phenotype of the cell in which it is expressed. Dysregulation of alternative splicing is a common occurrence in cancer and may lead to the formation of truncated or degraded proteins through the introduction of immature stop codons or nonsense mediated decay. Increasing evidence indicates that cancer-associated splicing variants play an important role in tumor initiation and progression. In this review, we summarize the evidence supporting the relevance of alternative splicing in glioblastoma multiforme (GBM). Specifically, we focus on the role of alternative splicing in GBM pathogenesis with an emphasis on the effect of aberrant alternative splicing of FGFR, GLI-1, and EGFR. The significance of exploiting alternatively spliced isoforms as potential biomarkers which may contribute to the development of diagnostic and prognostic methods, in addition to serving as molecular targets in GBM, will be discussed.

Keywords—glioblastoma; alternative splicing

INTRODUCTION

Alternative splicing is a key regulator of gene expression and enables the generation of numerous transcripts from a single gene, thereby increasing the coding potential of the genome. During mRNA processing, approximately 90% of pre-mRNA is removed as introns, with the remaining 10% joined together as exonic sequences (Tazi, Bakkour, and Stamm 2009). Thus the process has been considered a key driver in the evolution of phenotypic complexity. Splicing is the process by which introns are removed from a precursor mRNA (pre-2mRNA) and exons are ligated to form a mature mRNA. Considered one of the most complicated RNA- protein complexes within the eukaryotic cell (Nilsen 2003), pre-mRNA splicing is catalyzed by the spliceosome, a large complex composed of five small nuclear ribonucleoprotein (RNP) subunits each composed of a single uridine rich small nuclear RNA (snRNA) in addition to as many as 150 proteins that assemble in an ordered stepwise manner on each intron to be spliced [reviewed in (Kim, Goren, and Ast 2008, Smith, Query, and Konarska 2008, Wahl, Will, and Luhrmann 2009)]. The spliceosome performs the two primary functions of splicing: recognition of the intron/exon boundaries and catalysis of the cut and paste reactions that remove introns and join exons. It is responsible for directing both constitutive and alternative splicing (Faustino and Cooper 2003).

Definition of the exon/intron boundary is maintained through cis-regulatory RNA elements which serve as splicing enhancers or silencers, essentially determining the splice site. Splicing regulatory elements (SREs) fall into four different categories: exonic splicing enhancers (ESEs), intronic splicing enhancers (ISEs), exonic splicing silencers (ESSs), and intronic splicing silencers (ISSs) (Matera and Wang 2014). These SREs function by recruiting trans-acting splicing factors which can either activate or suppress different steps of the splicing reaction (Matera and Wang 2014). Additionally, splicing factors help regulate splicing by interacting with the core components of the spliceosome. Some common splicing factors include serine-arginine rich (SR) proteins which recognize ESEs to promote splicing, and heterogenous nuclear ribonucleoproteins (hnRNPs) which recognize ESSs to inhibit splicing. The activity of splicing factors and SREs is context-dependent with the most frequent form of alternative splicing being expression of a cassette exon (exon skipping). Other alternative splicing modes include mutual exclusivity, use of alternative 3' or 5' splice sites, alternate transcriptional start sites, and intron retention [Figure 1, (Tazi, Bakkour, and Stamm 2009)].

Dysregulation of splicing has been associated with several human diseases including spinal muscular atrophy, Alzheimer's disease, and Parkinson's disease (Zhang et al. 2008, Glatz et al. 2006, Fu et al. 2013), and its connection with cancer, resulting in the activation of oncogenes or inactivation of tumor suppressors, has been well established [reviewed in (Oltean and Bates 2014, Zhang and Manley 2013, David and Manley 2010)]. For example, the splicing factor SF2/ASF, up-regulated in a variety of cancer types, acts as a proto-oncogene by affecting the alternative splicing of BIN1, S6K1 and MNK2. Specifically, expression of SF2/ASF inhibited the tumor suppressor function of BIN1 by inclusion of exon 12A, induced expression of the oncogenic isoform 2 of S6K1, and increased the phosphorylation of eIF4E by inducing expression of MNK2b. Targeted inhibition of SF2/ASF by shRNA abolished NIH-3T3 cellular transformation and significantly reduced tumorigenesis *in vivo* (Karni et al. 2007).

In this review, we will discuss alternative splicing in glioblastoma multiforme (GBM), the most common and lethal primary tumor of the central nervous system. GBM is classified by the World Health Organization as a grade IV astrocytoma, and is characterized by the presence of tumor cell necrosis and/or microvascular proliferation, in addition to cytological atypia, anaplasia, and mitotic activity (Louis et al. 2007). The current standard of care for GBM patients includes concomitant and adjuvant temozolomide plus radiotherapy, and leaves patients with a median survival of only about 15 months from the time of diagnosis (Stupp et al. 2005, Weller et al. 2014). The dismal prognosis for GBM patients provides a strong incentive for further research; a better understanding of the molecular alterations in GBM, such as aberrant alternative splicing, may help contribute to the discovery of future therapies.

Importantly, the recent characterization of the genome and transcriptome of GBM has enabled the use of comprehensive bioinformatics approaches in the investigation of alternative splicing in GBM. For example, using the exon-level expression profile data from the Gene Expression Omnibus (GEO) database, Yu et al, identified 617 alternatively spliced genes in GBM, information which may provide novel molecular markers for the diagnosis and treatment of GBM (Yu and Fu 2014). In another report, Sadeque et al, related alternative exon usage in 25,403 genes to the survival of 250 GBM patients and found that in 2,477 genes alternative exon usage was significantly associated with patient survival (Sadeque et al. 2012). Here we provide an overview of the published work which has indicated a role for alternative splicing in the initiation and/or progression of GBM. Specifically, we will highlight the alternatively spliced isoforms of the fibroblast growth factor receptor 1 (FGFR1), the glioma-associated oncogene homolog 1 (Gli1), and the epidermal growth factor receptor (EGFR) as their contributions to the pathogenesis of GBM have been extensively characterized.

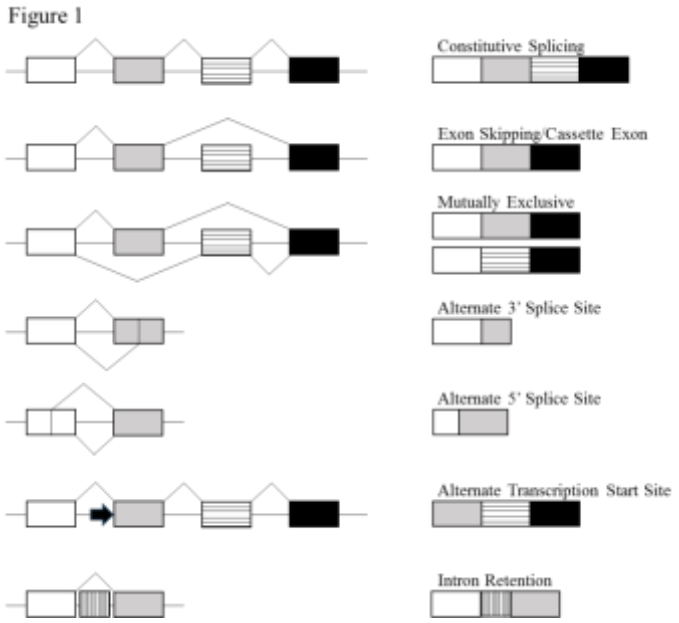

Figure 1. Modes of Alternative Splicing
Exons shown as boxes, introns as black lines. Arrow indicates alternate promoter. Splicing shown on left, resulting transcript on right.

1. Alternative Splicing in the Pathogenesis of Glioma

Tumorigenesis is a multistep process. It has been postulated that malignant growth is a manifestation of eight essential alterations in cell physiology which govern the transformation of normal human cells into malignant cancers: sustaining self-sufficiency, insensitivity to growth suppressors, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion, reprogramming of energy metabolism, and evasion of immune destruction (Hanahan and Weinberg 2000, 2011). More recently, it has been postulated that another fundamental characteristic of cancer cells, loss of terminal differentiation, should be considered a "hallmark" as well (Floor et al. 2012). It is becoming increasingly clear that aberrant alternative splicing plays a role in the regulation of many of these processes in GBM. In this section we present published examples of splicing isoforms relevant to some of these hallmarks of cancer that are functionally significant in the pathogenesis of GBM. Additionally, table 1 lists several examples of splice variants found to be present in GBM for which the function/mechanistic role is not yet characterized.

1.1 Alternative Splicing in GBM Proliferation and Invasion

One of the most fundamental traits of cancer cells involves their ability to sustain chronic proliferation. Attesting to uncontrolled cellular proliferation, there have been many examples from the literature indicating a role for alternative splicing in the proliferative capability of GBM. The first study we will examine describes the alternative splicing of KLF6 (Kruppel-like transcription factor). In this paper, authors demonstrate the existence of an allelic imbalance of KLF6 and its dominant negative splice isoform KLF6-SV1 in GBM. Here they show that the KLF6 tumor suppressor gene is reduced by ~80% in GBM, while KLF6-SV1 is significantly increased. Importantly, targeted KLF6-SV1 knock-down in A235 glioma cells significantly decreased their cellular proliferation (Camacho-Vanegas et al. 2007). This effect is likely due to KLF6-SV1 antagonizing the full-length KLF6 protein, as is the case in prostate cancer (Narla, Difeo, Reeves, et al. 2005, Narla, DiFeo, Yao, et al. 2005).

In another study, alternative splicing was shown to inhibit the tumor suppressor function of the KAP protein and increase cellular proliferation through the generation of a dominant negative variant. The cyclin-dependent kinase-associated protein phosphatase, KAP, functions to dephosphorylate Cdk2 and inhibit cell cycle progression (Hannon, Casso, and Beach 1994). Unexpectedly, increased KAP mRNA expression was found in GBM, and its expression correlated with increased histological grade and decreased patient survival (Yu et al. 2007). The paradoxical functioning of KAP in GBM was shown to be due to the aberrant alternative splicing of its transcript leading to the formation of several KAP mRNA splice variants containing deletions at exons 2 and 3. These splice variants were unable to generate the full length functional KAP protein and acted as dominant negatives against the full-length KAP, ultimately promoting GBM proliferation (Yu et al. 2007).

Having a role in several facets of tumorigenesis, alternative splicing of the human crk gene in GBM results in the aberrant expression of its isoform I. Crk was originally isolated as an oncogene product of the CT10 chicken retrovirus and has two alternatively spliced isoforms (Feller et al. 1998). While crkII is expressed in both normal cells and GBM, crkI is expressed at low levels in normal brain but is highly up-regulated in GBM. Authors compared the function of each isoform in glioma malignancy by transfecting U87 glioma cells with either CrkI or CrkII and examining the effect of each isoform on GBM phenotype. They found that expression of CrkI increased cellular transformation, migration, and invasion due to increased p130cas tyrosine phosphorylation and the ensuing activation of the FAK/p130cas/Crk/DOCK180 pathway (Takino et al. 2003).

The splicing factor, polypyrimidine tract-binding protein 1 (PTBP1/hnRNP I), plays a key role in alternative splicing by controlling the alternate 5' and 3' splice site usage (Hamid and Makeyev 2014). Binding adjacent introns or within exons themselves, PTBP1 represses the splicing of a large number of exons (Han et al. 2014). During development, PTBP1 and PTBP2 are expressed in glial and neuronal precursor cells respectively, and both transcripts decrease as cells differentiate to form the mature brain (Boutz et al. 2007). Importantly,

PTBP1 is aberrantly overexpressed in GBM and may serve as a marker of progression (Cheung et al. 2006). In this next study, authors investigated PTBP1 overexpression in GBM. They found that PTBP1 specifically represses exon 3 expression in the RTN4 (Nogo) gene, resulting in decreased proliferation and invasion of glioma cells (Cheung et al. 2009).

PTBP1 has also been shown to mediate the alternative splicing of cassette exon 6 in the membrane-binding tumor suppressor annexin A7 (ANXA7) (Ferrarese et al. 2014). Re-expression of exon 6 by inhibition of PTBP1 increased endosomal targeting of EGFR, reduced angiogenesis and invasion, and ultimately reduced the malignancy of GBM (Ferrarese et al. 2014). Interestingly, in another study, inhibition of GSK3β with the small molecule inhibitor AR-A014418 was shown to result in a time-dependent switch in alternative splicing from exon 6 exclusion to exon 6 inclusion in U87 and U373 glioma cells (Yadav et al. 2014). In this report, authors show a significant reduction in the splicing factors SRSF1, SRSF5, PTBP1, and hnRNPH3 upon GSK3 inhibition, likely mediating the ANXA7 alternative splicing switch. Although the direct targeting of aberrant alternative splicing will be discussed in greater length later in this review, this study suggests the importance of upstream effectors in alternative splicing and a mechanism for their subsequent inhibition.

Several reports have also linked alternative splicing of the tyrosine kinase receptor Recepteur d'Origine Nantais (RON) to GBM pathogenesis. RONΔ11 is a constitutively active isoform of the full length variant which lacks exon 11 and has been shown to promote cell motility and the epithelial-mesenchymal transition. Interestingly, exclusion of exon 11 has been attributed to at least two different regulatory splicing factors in GBM. Lefave, et al showed that RONΔ11 is significantly enhanced in GBM, and the overt exclusion of exon 11 was due to the regulatory splicing factor hnRNPH. HnRNPH was found increased in GBM and correlated with tumor grade. *In vitro*, its targeted inhibition decreased GBM invasiveness (Lefave et al. 2011). In another report, exon 11 skipping in RON was attributed to the splicing factor hnRNPA2/B1, which was also found to be significantly up-regulated in GBM and inversely correlated with patient survival (Golan- Gerstl et al. 2011). In this study, inhibition of RON expression in U87 glioma cells rescued hnRNPA2/B1-mediated increase in soft agar colony formation. The discrepancy in splicing factors mediating exon 11 exclusion may be due to the existence of multiple regulatory elements acting on the RON transcript, a collaboration between hnRNPH and hnRNPA2/B1, or an evolutionarily driven redundancy in the pathogenesis of GBM.

1.2 Alternative Splicing in Terminal Differentiation

Alternative splicing is often tissue-specific, and the production of multiple variants from a single gene frequently occurs in a developmental stage-specific manner. In the cancer stem cell concept, a small population of GBM cells behave similarly to normal stem cells in that they are able to self-renew and give rise to a variety of differentiated tumor cells (Campos et al. 2010). The RNA binding protein and splicing regulator A2BP1 (ataxin 2 binding protein 1, Rbfox1) is an alternative splicing factor that functions in neuronal development and is expressed exclusively in differentiated neurons. In this next study, authors show that A2BP1 is deleted and down-regulated in GBM and its loss contributes to tumorigenesis by compromising terminal differentiation (Hu et al. 2013). It was shown that A2BP1 regulates the mutually exclusive splicing of exons a and b in the tumor suppressor TPM1, maintaining a high exon b/exon a ratio, and driving the neuronal differentiation of neural progenitor cells. Ectopic expression of TPM1-6b significantly suppressed intracranial tumor formation (Hu et al. 2013). Results from this study indicate that the immature differentiation state in GBM may be partly driven by a neutralization in the alternative splicing mechanisms which contribute to terminal differentiation.

1.3 Alternative splicing in GBM Metabolism

The ability for cancer cells to alter their metabolism to cope with hypoxia was first described by Otto Heinrich Warburg in 1956 (Warburg 1956a, b). The Warburg effect describes the increased uptake and conversion of glucose to lactate by cancer cells under adequate oxygen conditions, essentially foregoing oxidative phosphorylation for aerobic glycolysis. Aerobic glycolysis is partly regulated by the expression of pyruvate kinase isoforms, and there are several lines of evidence indicating that alternative splicing of pyruvate kinase is a critical determinant of the Warburg phenotype in GBM.

Pyruvate kinase catalyzes the final step in glycolysis, generating pyruvate and ATP. It is encoded by two paralagous genes, PK-L and PK-M, which each give rise to two alternatively spliced isoforms, L and R, M1 and M2, respectively (Jurica et al. 1998). The differentiated isoform of PK-M, PK-M1, promotes oxidative phosphorylation while the embryonic isoform, PK-M2, promotes aerobic glycolysis; the mutually exclusive alternative splicing of exons 9 and 10 in the PK-M pre-mRNA results in expression of PK-M1 (exon 9 included) or PK-M2 (exon 10 included) (David and Manley 2010, Chaneton and Gottlieb 2012).

Recent evidence has indicated that PK-M2 confers a selective growth advantage to tumor cells (Li, Yang, and Li 2014, Christofk et al. 2008). In a study by Clower, et al authors showed that shRNA mediated knock-down of the splicing factors hnRNPA1, hnRNPA2, and PTBP1 resulted in a significant increase in the PK-M1 isoform expression and a decrease in lactate production in GBM cells (Clower et al. 2010). Interestingly, it has been shown that all three of the aforementioned alternative splicing factors, PTBP1, hnRNPA1, and hnRNPA2 are overexpressed in GBM and their expression is directly attributable to the transcription factor c-Myc (David and Manley 2010). That neoplastic cells primarily express PK-M2 over PK-M1 indicates a switch in the state of differentiation

of these cells. Indeed, Clower et al show that when proliferating myoblasts are induced to terminally differentiate, there is a resultant almost exclusive switch from PK-M2 to PK-M1. It was interesting to note that of all the cancer tissues analyzed for PK-M isoform expression, only the two GBM lines tested showed evidence of both isoforms. This contradiction may be attributable to the remarkable heterogeneity of GBM.

Linking the GBM mutant EGFRvIII with alternative splicing in cellular metabolism, Babic, et al recently showed that expression of EGFRvIII in U87 glioma cells significantly increased the expression of hnRNPA1 (Babic et al. 2013). In this report, they show that hnRNPA1 mediates the alternative splicing of the myc-interacting protein Max, generating a truncated variant, Delta Max, whose overexpression increased GBM cell proliferation and promoted glycolytic gene expression in a Myc-dependent fashion (Babic et al. 2013). As EGFRvIII functionally synergizes with wtEGFR, it would be interesting to examine the ratio of PK-M1 to PK-M2 in GBM cells expressing both receptor types. It is possible that expression of PK-M1 in a subset of GBM cells confers a selective advantage to the tumor as a whole.

1.4 Alternative Splicing in Apoptosis

So far we have discussed aberrant splicing in the generation of variants which contribute to multiple aspects of GBM pathogenesis including tumor establishment, progression, and maintenance. In this next section we will discuss the role of aberrant alternative splicing in the molecular control of apoptosis, another hallmark of cancer. The death-domain adaptor protein Insuloma-Glucagonoma protein 20 (IG20) is consistently spliced to generate an antagonistic, anti- apoptotic isoform termed MADD (MAP-kinase activating death domain protein), which has exon 16 excluded. In this next study, authors show that while in normal brain there is equal expression of both IG20 and MADD isoforms, in GBM there is a switch from exon 16 inclusion to exon 16 exclusion forcing the expression of MADD. MADD has been shown to redirect TNFa/TRAIL- induced death signaling to promote survival and proliferation instead of triggering apoptosis (Mulherkar, Prasad, and Prabhakar 2007). In this report, it is shown that exon 16 exclusion is mediated by the splicing factor hnRNPH, which is also upregulated in GBM (Lefave et al. 2011). Indeed, siRNA mediated inhibition of hnRNPH resulted in increased exon 16 inclusion along with an increase in apoptosis and reduction in cell viability, indicating the therapeutic potential of MADD neutralization (Lefave et al. 2011).

In addition, there have been several reports describing the up-regulation of the apoptosis inhibitor protein survivin and its isoforms survivinΔEx3, survivin2α, survivin2B, survivin3α, and survivin3B in GBM. Virtually absent from normal differentiated cells, survivin is expressed in highly proliferative areas in normal tissues, including neural stem cells, and is expressed in virtually every human tumor (Caldas et al. 2005). The splice isoform survivinΔEx3, which lacks exon 3, has been shown to heterodimerize with survivin, and co-expression of these two variants protects medulloblastoma cells from apoptosis greater than expression of either variant alone (Caldas et al. 2005). Importantly, the ratio of survivinΔEx3 to survivin was found to be significantly higher in malignant brain tumors, including GBM, compared to benign ones, and expression of survivin, survivin2α, and survivin2B increased with tumor grade (Yamada et al. 2003, Huang et al. 2011).

We have previously discussed the work by Golan-Gersti, et al who described the up- regulation of the hnRNPA2/B1 splicing factor in GBM (Golan-Gerstl et al. 2011). In this same report, authors also revealed a significant effect of hnRNPA2/B1 on the alternative splicing of caspase 9. They show that with high expression of hnRNPA2/B1 there is a significant increase in the exclusion of exons 3-6 in caspase 9, effectively forming the truncated mutant caspase-9b. Caspase-9b, acting as a dominant negative, blocks the activation of caspase-9 and -3 effectively inhibiting apoptosis and its expression in multiple cancer types has been reported (Srinivasula et al. 1999).

Table 1. Glioma Specific Isoforms
Isoforms identified specifically in GBM, with yet unknown or uncharacterized functional relevance.

Gene	Splice Mechanism	Splice Variant	Function	Reference
MDM4	Exon Exclusion: 8, 9	MDM4-b	Negative regulator of p53	(Wang et al. 2013)
MDM2	Exon Exclusion: ND	MDM2-splice	Negative regulator of p53	(Kraus et al. 1999)
MDM2	Exon Exclusion: ND	MDM2-b	Negative regulator of p53	(Matsumoto et al. 1998)

SGK1	Alternate TSS: Exon 1 Intron 3	 SGK1.1 SGK1.2	Regulator of transport, cell volume, and cell survival	(Simon et al. 2007, Arteaga et al. 2008)
RFX4	Exon Exclusion: 1, 6 Alternate TSS: ND	 RFK4-D RFX4-E RFX4-F	Transcription Factor	(Matsushita et al. 2005)
IIp45	Exon Exclusion: 7	 IIp45S	Anti-invasion	(Song et al. 2005)
CALD1	Exon Inclusion: 1 1+4 1'+4	 1 1 + 4 1' + 4	Cytoskeleton-associated protein	(Zheng, van der Weiden, and Kros 2005, Zheng et al. 2004)
Ca(v)3.1	Exon Inclusion: 25a, 26	 Ca(v)3.1ac	Calcium Channel	(Latour et al. 2004, Bertolesi et al. 2006)
VEGF	Exon Exclusion: 6a, 6b, 7 6a, 6b 6b	 VEGF121 VEGF165 VEGF189	Angiogenesis	(Huang et al. 2005, Cheng et al. 1997)
MAP-2	Exon Inclusion/ Exclusion: 13/7a-8	 MAP-2e	Development	(Suzuki et al. 2002)
HdynIV	Exon Exclusion: 15	 HydnIV-26	Endocytosis and Vesicle Trafficking	(Chen et al. 2000, Chen et al. 2003)
V1	Exon Inclusion: 7	 V0	Chondroitan sulfate proteoglycan	(Dours-Zimmermann and Zimmermann 1994)
RTVP-1	Exon Inclusion: 71bp deletion between 2 & 3	 RTVP-1b	Pathogenesis	(Xiang et al. 2007)
RSU-1	Exon Exclusion: 133bp deletion	Exon-deleted RSU-1	Tumor suppressor	(Chunduru et al. 2002)

Abbreviations—MDM, mouse double minute; KLF, Krüppel like factor; SGK, serum- and glucocorticoid-inducible kinase 1; ADAM, a disintegrin and metalloproteinase domain; RFX4, Regulatory Factor X4; IIp45, Invasion Inhibitory Protein 45; CALD1, Caldesmon; Ca(v)3.2, T- type calcium-channel; VEGF, Vascular Endothelial Growth Factor; MAP-2, Microtubule- associated protein-2; HdynIV, Dynamin-like protein; V1, versican; RTVP, related to testes- specific, vespid, and pathogenesis protein; RSU-1, Ras suppressor protein 1, ND, Not Determined; TSS, Transcription Start Site

2. Alternative Splicing of FGFR

Alternative splicing leading to the differential expression of the fibroblast growth factor receptor (FGFR) has been shown to play a critical role in the malignant progression of GBM. The FGFR family is composed of four different receptors, FGFR1 – FGFR4, with several alternatively spliced variants of FGFR1 and FGFR2 (Werner et al. 1993). Increased expression of FGFR1 in GBM is often accompanied by a shift in its splicing pattern, with FGFR1β as the predominant isoform (Yamaguchi et al. 1994). Compared to FGFR1α, FGFR1β, lacking exon 3 (alpha exon), has an increased affinity for ligand due to the absence of one of the three extracellular NH2- terminal loops and its expression results in a cell-growth advantage, contributing to GBM malignancy (Yamada et al. 1999, Wang,

Kan, Yan, et al. 1995, Wang, Kan, Xu, et al. 1995, Loilome et al. 2009).

A good deal of progress has been made in understanding the molecular mechanisms behind the aberrant alternative splicing switch from FGFR1α to FGFR1β since its discovery. Interestingly, FGFR1 contains two repressor elements capable of inhibiting exon 3 recognition, one downstream and one upstream, which are functionally redundant and exhibit significant sequence homology (Jin et al. 1998, 1999). The splicing factor PTBP1, whose expression is strongly increased in tumor tissue compared to adjacent normal brain, has been shown to be one of the regulators of FGFR1 splicing by specifically binding the ISS-1 regulatory element in the intron upstream of exon 3 (Jin et al. 2000). Use of antisense RNA oligonucleotides to target the ISS-1 regulatory element inhibited PTBP1 binding and enhanced endogenous exon 3 inclusion (Jin et al. 2003).

Importantly, another study in which authors used antisense morpholino oligonucleotides to target the ISS flanking exon 3 of FGFR1 described an increase in caspase-3 and -7 in U251 glioma cells, without affecting cell viability (Bruno, Jin, and Cote 2004). It is noteworthy that use of the oligonucleotides increased exon 3 inclusion from 10% to 70% *in vivo*, making this a strong therapeutic candidate for use in GBM patients. The use of oligonucleotides as a therapy for targeting alternative splicing events will be discussed more thoroughly later in this review.

3. Alternative Splicing of GLI-1

The glioma-associated oncogene homologue 1 (GLI1) is a member of the GLI transcription factors which are the main effectors of the Sonic Hedgehog (shh) pathway. Addition of shh ligand to cells, activates a signaling cascade leading to the translocation of GLI to the nucleus enabling the transcription of several genes including GLI1 (Carpenter and Lo 2012, Cui et al. 2010). GLI1 was first identified in 1987 as it was highly upregulated in a GBM tumor (Kinzler et al. 1987). Since then, it has been shown that GLI1 undergoes alternative splicing resulting in the expression of several different splice variants, including GLI1ΔN and tGLI1 (Wang and Rothnagel 2001). While GLI1ΔN is prevalent in both normal and cancerous tissues and behaves similarly to the full length GLI1, tGLI1, although absent in normal tissue, is highly expressed in GBM and other cancers (Carpenter and Lo 2012, Lo et al. 2009, Shimokawa et al. 2008).

The tGLI1 isoform is a truncated splice variant with exon 3 and part of exon 4 deleted (Lo et al. 2009, Zhu et al. 2014). While tGLI1 undergoes nuclear translocation similar to full-length GLI1 (GLI1FL), there are more than 100 genes differentially expressed between the two. Importantly, tGLI1 was shown to bind CD24 with a significantly greater affinity than GLI1, and the subsequent transcriptional activation of CD24 by tGLI1 increased the migration and invasion of U87 GBM cells (Lo et al. 2009). Furthermore, expression of tGLI1 has been directly linked to enhanced tumor growth and vascularity of GBM xenografts (Zhu et al. 2014). In this report, authors show that tGLI1-expressing GBM xenografts were more aggressive in growth, with higher microvascular density, and increased Ki-67 positive cells than in the control. Further supporting its role as a novel mediator of neoangiogenesis, tGLI1-expressing tumor cells strongly up- regulated VEGFA and heparanase promoting increased tubule formation of vascular endothelial cells and increased the angiogenesis of chick embryos *in vivo* (Zhu et al. 2014). Interestingly, GLI1FL has been shown to mediate resistance to chemotherapy in GBM, however the effects of GLI1 variants in this realm have not yet been reported (Cui et al. 2010).

4. Alternative Splicing of EGFR

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein whose dimerization upon ligand binding results in its autophosphorylation and activation (Ogiso et al. 2002). While amplification of EGFR is the most common genetic aberration associated with GBM, it is also among the most frequently mutated genes and maintains a diversity of altered transcripts. The most common mutant form associated with GBM is EGFRvIII, although a variety of other recurrent non-canonical EGFR transcript forms have been detected. In a recent report, 57% of 164 RNA samples from GBM patients showed evidence of mutation, rearrangement, altered splicing and/or focal amplification of EGFR (Brennan et al. 2013). Interestingly, nearly all EGFR mutations have been detected in tumors with EGFR amplification and multiple types of EGFR mutations can be detected in individual tumors attesting to the heterogeneity of GBM (Pines et al. 2010, Frederick et al. 2000).

The EGFR gene is located at chromosome 7p12 and contains 28 exons transcribed into a protein with three distinct domains: extracellular (ECD), transmembrane (TMD), and intracellular (ICD). There are five common mutants, EGFRvI – EGFRvV. Mutants EGFRvII, EGFRvIII and EGFRvIV have entire exons deleted, while EGFRvI and EGFRvV have NH2-terminal and COOH- terminal truncations, respectively (Figure 2) (Nicholas et al. 2006). EGFRvI and EGFRvIV are rare, while EGFRvII and EGFRvV are marginally more common, each accounting about 10% of all GBM-associated EGFR mutations (Kastenhuber et al. 2014). While functional analyses of mutant EGFRvIII have been well-characterized, the same cannot be said for other EGFR mutants, likely attributable to their frequent co-occurrence with EGFRvIII. However, some studies have begun to tease apart the functional relevance of each mutant, and these are summarized below.

We will begin by discussing the EGFR ECD mutants EGFRvI, EGFRvII, and EGFRvIII. EGFRvI has an extensive N-terminal truncation comprising almost the entire extracellular domain. Resembling the viral erbB oncogenic protein, in GBM this mutant is constitutively active independently of ligand (Voldborg et al. 1997, Humphrey et al. 1988, Wong et al. 1992), however the functional relevance of this variant in GBM pathogenesis remains unclear.

The EGFRvII mutant bears an in-frame deletion from amino acids 520-603 within domain IV of the ECD. This mutant is capable of high affinity ligand binding, resulting in its activation. It responds to growth factors in a similar fashion as wtEGFR: treatment with EGF results in enhanced tyrosine kinase autophosphorylation, increasing cellular proliferation and invasion *in vitro* (Humphrey et al. 1991). However, its constitutive phosphorylation, indicative of ligand- independent activation, has also recently been described (Francis et al. 2014). Indeed, expression of the EGFRvII variant in E14.5 neural stem cells revealed its constitutive phosphorylation along with an increased capacity to form subcutaneous tumors in nude mice when compared to cells expressing wtEGFR (Francis et al. 2014).

By far the most common mutant, EGFRvIII arises through an in-frame deletion of 801 bp from the EGFR ECD due to the genomic deletion of exons 2-7. Its effects on glioma pathogenicity have been well-characterized, and are primarily attributable to its constitutively low level of activation and defective receptor internalization (Huang et al. 1997). The effect of EGFRvIII expression in promoting the tumorigenicity of GBM xenografts has been extensively documented. Originally attributed to an increase in proliferation along with a reduction in apoptosis due to an up-regulation of Bcl-XL, EGFRvIII has since been shown to stimulate GBM invasion, play key roles in mediating chemo- and radio-therapy resistance in GBM, and contribute to GBM heterogeneity (Nishikawa et al. 1994, Nagane et al. 1996, Nagane et al. 1998, Nagane et al. 2001, Lu et al. 2009, Inda et al. 2010, Nathanson et al. 2014, Feng et al. 2014).

Less frequently mutated in GBM, two EGFRvIV mutants have been described with deletions in the ICD: EGFRvIVa which lacks exons 25-27 and EGFRvIVb which lacks exons 25 and 26 (Kuan, Wikstrand, and Bigner 2001). The EGFRvIV mutants show constitutive dimerization and autophosphorylation, independently of ligand. Subcutaneous implantation of U87 cells expressing either EGFRvIV, EGFRvIII or wtEGFR in mice, revealed an increase in tumorigenicity in cells expressing EGFRvIV compared to wtEGFR, although to a lesser extent than EGFRvIII (Pines et al. 2010).

The mutant EGFRvV is a C-terminal truncated EGFR which has been reported to show increased ligand-dependent kinase activity and signaling along with a deficiency in internalization (Chen et al. 1989). Additionally, it maintains a much broader substrate specificity than wtEGFR (Decker, Alexander, and Habib 1992).

Cho et al, recently characterized the prevalence and oncogenic potential of EGFR C- terminal domain (CTD) mutants, including EGFRvIV and EGFRvV. An analysis of 469 GBM samples from the TCGA data for the presence of C-terminal exonic deletions confirmed the presence of three different C-terminal deletions of EGFR: exons 25-28 (EGFRvV), exons 25-27 (EGFRvIV), and exon 27 (Cho et al. 2011). Forced expression of each EGFR-CTD mutant in Ba/F3 cells promoted IL3 independent growth, demonstrating oncogenic function and EGF ligand-independent receptor autophosphorylation. Importantly, expression of EGFR-CTD mutants in LN443 GBM cells significantly increased their tumorigenicity in an intracranial mouse model compared to control (Cho et al. 2011).

While the EGFRvI-vV mutants have been the most frequently reported, many other mutants exist due to missense, duplication-insertion or other in-frame deletions. For example, EGFRvVI and EGFRvVII are EGFR mutants that are composites of EGFRvIII plus EGFRvIV or EGFRvV deletions, and the existence of a double mutant EGFRvIII with an additional deletion at exons 12-13, EGFRvIII/Δ12-13, has been reported (Kuan, Wikstrand, and Bigner 2001). Additionally, the existence of a mutant EGFR harboring a deletion from exons 13-16, denoted EGFRvII-extended, has recently been described (Francis et al. 2014). It has been hypothesized that alternative splicing may play a role in the expression of these mutant EGFR variants. For example, sequencing cDNA from the EGFR mutant deleted for exon 27 confirmed transcription of an aberrant mRNA, consistent with splicing between exons 26 and 28 (Cho et al. 2011). Future studies are warranted to determine to which splicing factor this may be attributed.

Aside from the common EGFR mutants, the existence of alternative EGFR transcript mRNAs has been described (Reiter et al. 2001, Reiter and Maihle 1996). For example, Piccione et al describe the identification and characterization of mLEEK, a novel EGFR variant deleted at exons 2-22 and overexpressed in GBM (Piccione et al. 2012). siRNA-mediated knock-down of mLEEK dramatically reduced cell viability corresponding to a significant induction in caspase-3 and -7 activity. It was reported that mLEEK is produced in the absence of genomic rearrangement and amplification, therefore alternative splicing may be attributable to its formation (Piccione et al. 2012). Along these same lines, EGFRvIII has been detected in a number of non-glioma tumors devoid of gene amplification or rearrangement and it has been suggested that alternative splicing also contributes to its formation (Okamoto et al. 2003). Specifically, the EGFRvIII mutant has been predicted to occur through the splice joining of exons 1-8 (Pedersen et al. 2001, Sugawa et al. 1990, Schwechheimer, Huang, and Cavenee 1995). Another example which designates alternative splicing in the formation of EGFR variants is in the case of the EGFR variant de4 EGFR. In this report, de4 EGFR was identified as an EGFR mutant with an aberrant splice joining of exons 3 and 5 and was expressed in 4/40 glioma tissues examined (Wang et al. 2011). Similar to other EGFR mutants, de4 EGFR is intrinsically constitutively activated with a low basal tyrosine phosphorylation due to its ability to self-dimerize in the absence of ligand. Overexpression of de4 EGFR in U87 glioma cells resulted in increased proliferation, anchorage-independent colony formation, migration, and invasion (Wang et al. 2011).

All of the EGFR mutants discussed thus far exist as transmembrane proteins. Soluble EGFR (sEGFR) isoforms are comprised solely of the extracellular domain, and are genuine

variants generated by alternative splicing. Specifically, sEGFR isoforms b, c, and d are encoded by the EGFR mRNA variants v2, v3, and v4 (Guillaudeau, Durand, Bessette, et al. 2012). These isoforms have been detected in both normal and malignant cells including adenocarcinoma, breast cancer, and ovarian cancer (Perez-Torres et al. 2008, Baron et al. 2009). And more recently EGFRv2, v3, and v4 transcripts were shown to be expressed in GBM in addition to the full length wtEGFR and mutant EGFRvIII. Although their functional relevance in GBM has not yet been investigated, these variant mRNA transcripts were significantly increased in GBM tissues which had wtEGFR amplification (Guillaudeau, Durand, Rabinovitch-Chable, et al. 2012).

Figure 2

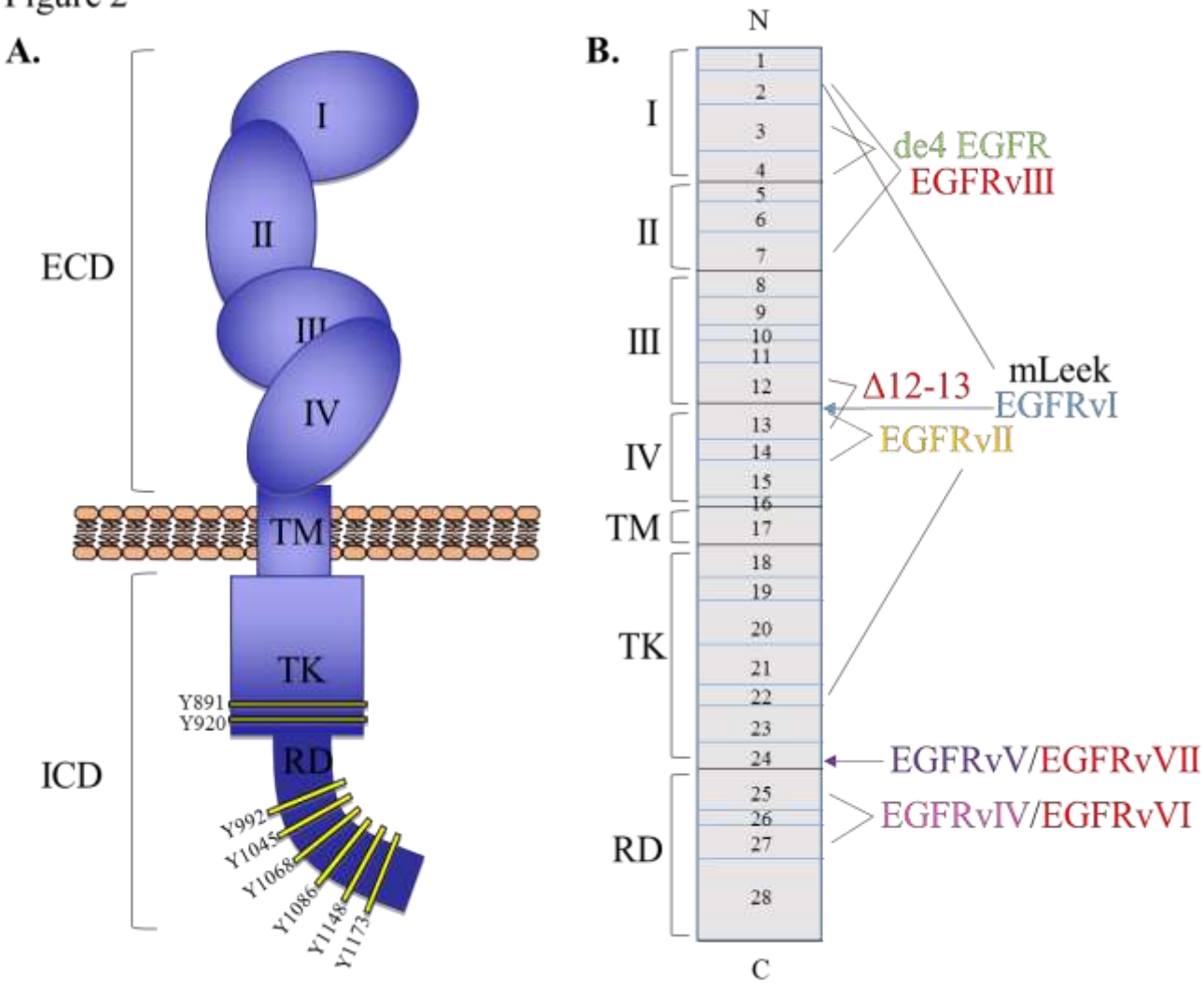

Figure 2: EGFR Organization and Mutants
A. Schematic depicting the EGFR protein at the cell surface. Y891-Y1173 indicate tyrosine phosphorylation sites.
B. Location of mutants within the EGFR protein composed of exons 1-28. Deletion mutants co-occurring with EGFRvIII are in red. de4 EGFR – exon 3-5 splice joining; EGFRvIII – exon 2-7 deletion; mLEEK – exon 2-22 deletion; EGFRvI – N-542 NH_2- terminal truncation; EGFRvII – exon 14-15 deletion; EGFRvV – C-958 COOH-terminal truncation; EGFRvIV – exon 25-27 deletion. EGFRvVII – EGFRvIII + EGFRvV composite; EGFRvVI – EGFRvIII + EGFRvIV composite; EGFRVIII/Δ12-13 – EGFRvIII + exon 12-13 deletion. I, II, III, and IV indicate subdomains. ECD: Extracellular Domain, ICD: Intracellular Domain, TM: Transmembrane, TK: Tyrosine Kinase, RD: Regulatory/Phosphorylation Domain.

5. Alternatively Spliced Variants as Therapeutic Agents

The exploitation of aberrant alternatively spliced variants for use as novel therapeutic agents against GBM has been well-documented. In this section, we will describe several studies in which alternatively spliced variants, acting as dominant negatives, have been utilized as a therapeutic modality. We will also discuss the use of antisense oligonucleotides and monoclonal antibodies that specifically target alternatively spliced isoforms.

The Receptor for Advanced Glycation End-products (RAGE) is a multi-ligand receptor expressed at high levels in GBM (Tafani et al. 2011). Its expression leads to the activation of several downstream signaling pathways involved in cell proliferation, survival, differentiation, migration, phagocytosis, and autophagy (Chen et al. 2014). Previous studies have shown that the ICD of RAGE is essential for its activation. Indeed, Taguchi et al, have shown that ectopic expression of a truncated RAGE mutant lacking the cytosolic tail acts as a dominant negative against RAGE signaling (Taguchi et al. 2000). In this report, expression of the tail-deleted mutant RAGE in C6 glioma cells resulted in a significant suppression of cellular proliferation, invasion, migration, and tumorigenicity. While the alternative splicing of RAGE has been extensively documented (Park et al. 2004, Hudson et al. 2008), only recently has an endogenous truncated RAGE (RAGEΔICD) been identified. Ectopic expression of the RAGEΔICD splice variant in C6 glioma cells reduced MAPK signaling, decreased cell motility, and decreased invasiveness compared to cells expressing the full length RAGE (Jules, Maiguel, and Hudson 2013). Additionally, cells expressing RAGEΔICD showed a reduction in proliferation and anchorage- independent colony formation compared to control. This study reveals the existence of an endogenously spliced truncated RAGE isoform which acts as a dominant negative against the tumorigenic effects of full-length RAGE.

The endogenous truncated variant of scatter factor/hepatocyte growth factor (SF/HGF), referred to as HGF/NK2, is another example of an alternatively spliced variant which may be exploited to antagonize growth factor signaling in GBM. SF/HGF, which binds the receptor c-met, has been shown to play key roles in glioma pathogenesis, such as increasing invasion, migration, inflammatory cell recruitment, and angiogenesis (Hamasuna et al. 1999, Badie et al. 1999, Lamszus et al. 1998, Moriyama et al. 1998, Laterra et al. 1997). Secretion of its splice variant HGF/NK2 has been projected to function in a dominant negative manner through its high-affinity binding to the c-met receptor, disrupting the autocrine signaling of SF/HGF (Chan et al. 1991). Indeed, investigating the effect of HGF/NK2 expression in GBM malignancy, authors show that its expression in U87 glioma cells inhibits colony formation and reduces migration *in vitro*, while reducing intracranial growth *in vivo* (Guerin et al. 2000).

In another example, the heparanase splice variant 36, originally cloned from the subterranean blind mole rat, Spalax, was also shown to act as a dominant negative to wild type heparanase. Heparanase is highly expressed in GBM and its overexpression results in a significant increase in migration, invasion, proliferation, and anchorage-independent colony formation in U251 glioma cells (Hong et al. 2010, Hong et al. 2008). Interestingly, expression of splice variant 36 in U87 glioma cells subcutaneously implanted in mice resulted in a significant reduction in tumor formation compared to control (Nasser et al. 2009).

The differential potency of alternatively spliced Bim variants is exemplified in a study by Yamaguchi et al, who found that the splice variant BimS was more effective at combatting GBM through gene therapy than BimL or BimEL. Bim is a pro-apoptotic BH3-only member of the Bcl-2 superfamily which is spliced into several different isoforms, including BimEL, BimL and BimS

(Yamaguchi et al. 2003). Of these, BimS is considerably more cytotoxic as it does not bind LC8, a component of the microtubule-associated dynein motor complex, and is therefore free to neutralize anti-apoptotic Bcl-2 members (Puthalakath et al. 1999). Indeed, glioma cells infected with adenovirus vectors expressing BimS (AVC2-BimS) were significantly more apoptotic than cells infected with AVC2-BimL or AVC2-BimFL. Furthermore, the combined injection of AVC2- BimS with AVC2-tk (an adenovirus expressing the herpes simplex virus thymidine kinase) markedly increased the sensitivity of U251 glioma cells to ganciclovir, as indicated by reduced *in vivo* tumor growth (Yamaguchi et al. 2003). The results from this study reveal the possibility of exploiting alternatively spliced variants in gene therapy for GBM.

Next we will discuss the use of antisense oligonucleotides and monoclonal antibodies to target specific protein variants which are aberrantly expressed and contribute to the pathogenesis of GBM. We previously discussed the aberrant alternative splicing of pyruvate kinase in GBM wherein exclusion of exon 9 generates the embryonic isoform PK-M2 important in aerobic glycolysis and tumor growth (Clower et al. 2010). Using an antisense oligonucleotide which specifically targets a novel enhancer in exon 10, authors of this next study were able to experimentally induce a switch in the splicing of endogenous PK-M transcripts to include exon 9. Inclusion of exon 9 down-regulated the expression of PK-M2 and increased apoptosis in GBM cell lines (Wang et al. 2012).

In another study, use of an antisense morpholino oligonucleotide treatment effectively reversed the aberrant splicing of USP5 by PTBP1. In this study, USP5 was identified as a novel target of PTBP1 whose increased expression, as in GBM and discussed previously (Cheung et al. 2009, Han et al. 2014), resulted in the aberrant generation of USP5 isoform 2. Using an antisense morpholino oligonucleotide which targets the 5' splice site of exon 15 in USP5 resulted in a dramatic switch from isoform 2 to isoform 1 in both U251 and LN229 GBM cell lines. Importantly, the forced expression of USP5 isoform 1 inhibited glioma cell growth and migration indicating an important role for USP5 splicing in GBM pathogenesis (Izaguirre et al. 2012). The results from these studies reveal the

potential for antisense morpholino oligonucleotides as a novel therapy for GBM.

Lastly, the generation of recombinant antibodies to specifically target splice variants involved in GBM pathogenesis has been a novel approach to selectively target tumor cells. Monoclonal antibodies (mAbs) targeting splice isoforms of Tenascin-C have been used in the clinic to deliver radioactive isotopes to GBM tumors for diagnostic and therapeutic purposes (Zalutsky et al. 1989). Tenascin-C is an extracellular matrix glycoprotein whose primary transcript is alternatively spliced, resulting in the generation of two main isoforms, small and large, with distinct biological functions. Specifically, the large isoform, which has been shown to confer loss of focal adhesion in cultured cells, is highly expressed in GBM and two mAbs, BC-2 and 81C6, which both specifically target the large isoform, have shown promise in the clinic (Borsi et al. 1994, Carnemolla et al. 1999, Reardon et al. 2002). Other anti-tenascin-c antibodies have been generated which may have greater selectivity for GBM tumor tissues including TN11, which specifically recognizes the type III repeat C of the large Tenascin-C isoform, and SIP(F16), specific to the alternatively spliced domain A1 of tenascin-C (Carnemolla et al. 1999, Brack et al. 2006).

Along these same lines, the mAb 806 specifically targets the mutant EGFRvIII and has shown significant anti-tumoral activity in human xenograft mouse models. Treatment of mice bearing intracranial EGFRvIII expressing U87 gliomas with mAb 806, resulted in a significant reduction in EGFRvIII autophosphorylation, decreased angiogenesis, and increased apoptosis compared to untreated tumors (Mishima et al. 2001). Importantly, these effects have been shown to be specific to cells expressing EGFRvIII or amplification of the EGFR gene, as mAb 806 had no bearing on normal wtEGFR expressing cells (Luwor et al. 2001). Use of a mAb 806 chimera in a Phase I clinical trial for patients with diverse tumor types revealed it was well-tolerated and specifically targeted the tumor site (Scott et al. 2007). Several other Phase I clinical trials measuring the safety, pharmacokinetics, biodistribution and imaging characteristics of mAb ABT-806 and ABT-806i, an indium-labeled conjugate of ABT-806, are currently underway (www.clinicaltrials.gov).

Administration of mAbs targeting tumor-specific isoforms expressed by GBM is an innovative therapeutic strategy that should be further examined in the clinic. Taken together, these studies indicate the potential to harness alternative splicing as a novel means to control oncogenic function, combatting GBM.

CONCLUSIONS

Alternative pre-mRNA splicing is an important mechanism in gene regulation. The alteration of alternative splicing in GBM may be used in the identification and characterization of glioma-specific splice variants that can be used as novel diagnostic and prognostic tumor biomarkers and potential targets for therapy. That aberrant alternative splicing is so widely recognized in cancer biology, indicates that it may drive potential escape mechanisms in the cancer cell. For example, compared to cells maintained in normal oxygen, T98G glioma cells maintained in a low oxygen environment show increased expression of the alternatively spliced isoform of the human prion protein (PrP), with a concomitant decrease in the normally spliced PrP transcript (Kikuchi et al. 2008). Further studies are warranted to investigate a role for alternative splicing in mechanisms of escape which may drive therapeutic resistance and tumor relapse.

The potential to reverse alternative splicing, in the absence of deletion, suggests a response to extracellular cues, enabling the adaptation of cells to changing environmental conditions. Several of the studies we discussed, and many of the variants listed in table 1, reveal a correlation between alternative splicing and tumor grade. One may postulate then that as a tumor grows, increasing in vascularity, with expanding hypoxic niches and further invading the brain, the ever- changing tumor microenvironment contributes to a disruption in the normal mRNA splice pattern. The remarkable heterogeneity of GBM may indeed be a facet of environmentally induced alternative splicing. It is interesting to hypothesize a role for alternative splicing in the generation of many different transcripts from the same gene, whose protein expression is effected simply by the location of the cell within which it resides. It is important to keep in mind, however, that it is the aberrant expression of splicing factors which generate aberrant splice forms. Ultimately a more thorough understanding of the regulation of these factors will be necessary to truly define a global role for alternative splicing in GBM.

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