Genomic Analysis of the BMP Family in Glioblastomas

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ABSTRACT: Glioblastoma multiforme (GBM) is a grade IV glioma with a median survival of 15 months. Recently, bone morphogenetic protein (BMP) signaling has been shown to promote survival in xenograft murine models. To gain a better understanding of the role of BMP signaling in human GBMs, we examined the genomic alterations of 90 genes associated with BMP signaling in GBM patient samples. We completed this analysis using publically available datasets compiled through The Cancer Genome Atlas and the Glioma Molecular Diagnostic Initiative. Here we show how mRNA expression is altered in GBM samples and how that is associated with patient survival, highlighting both known and novel associations between BMP signaling and GBM biology.

KEYWORDS: Glioblastoma multiforme, bone morphogenetic protein, genomics, The Cancer Genome Atlas, REMBRANDT

Introduction

Tumors in the brain known as gliomas are comprised of cells that resemble mature glial cells. These tumors include astrocytomas, oligodendrogliomas, ependymomas, and mixed gliomas.[1] Gliomas account for approximately 30% of all central nervous system (CNS) tumors and 80% of all malignant CNS tumors.[2] The World Health Organization (WHO) has classified astrocytomas into four grades based on histological features that correlate with patient outcomes.[3] Grade I and II tumors (or low-grade gliomas) are relatively slow-growing tumors that show low proliferative activity. Grade III and IV (or high-grade astrocytomas) are rapidly growing, highly infiltrative tumors that show anaplasia and mitotic activity. Grade IV astrocytomas show, in addition, microvascular proliferation and/or necrosis.[3] Approximately 17,000 malignant gliomas are diagnosed each year.[4] Glioblastoma multiforme (GBM), a grade IV astrocytoma, is the most common malignant CNS tumor, accounting for approximately 80% of malignant gliomas.[2,4] The survival rate for patients with GBM is extremely low, with less than 5% of patients surviving 5 years after diagnosis and a median survival of only 15 months despite surgical resection, radiation, and chemotherapy.[2,5] One of the primary challenges in finding effective therapies is due to the high level of heterogeneity found within GBMs. Although GBMs are diagnosed histopathologically as one disease, recently it has become clear that GBMs are heterogeneous at the molecular level.

Genetic Heterogeneity

GBMs are well known for their histologic heterogeneity. Recently, with the rapid advancement of sequencing technology and accumulation of genomic data, emphasis has been placed on understanding these tumors at the molecular genetic level. Such studies suggest that, while many GBMs share certain genetic and epigenetic alterations, tumors segregate into subclasses based on gene expression analysis. In 2006, Phillips et al established three subclasses of high-grade glioma (HGG) based on large-scale genomic expression profiles of survival-related genes. These subgroups are termed proneural (PN), proliferative, and mesenchymal (MES), reflecting the dominant gene expression patterns in each group.[6] In 2010, Verhaak et al classified HGGs into four similar subclasses using data compiled by The Cancer Genome Atlas (TCGA).[7] These subgroups show gene expression patterns similar to those found within neural lineages.[6] Phillips et al likened the gene expression signatures to various stages of neurogenesis in the adult forebrain. Thus, gene expression patterns of MES tumors are akin to that of undifferentiated neural stem cells, PN gene expression is similar to neuroblasts or immature neurons, and proliferative gene expression is reminiscent of transit-amplifying cells.[6] Phillips et al further provided evidence of GBM subclass switching, exclusively to the MES pattern, upon tumor recurrence. These data suggest that a better understanding of the pathways regulating neural development is crucial to understand the underlying signaling

pathways and networks that give rise to GBM subtypes and may regulate the phenomenon of subclass switching.

Bone Morphogenetic Protein Pathway

One of the most important signaling pathways regulating neural development is the bone morphogenetic protein (BMP) pathway. The BMP signaling pathway is part of the transforming growth factor beta family (TGFβ). The TGFβ family has more than 30 members divided into families of activins, nodals, growth differentiation factors (GDFs), and BMPs.[8] As with other members of the TGFβ family, during canonical signaling, BMP ligands bind to type I and type II serine-threonine kinase receptors, which transduce the signal downstream through phosphorylation of specific regulatory Smad proteins. During canonical BMP signaling, BMP ligands bind to the BMP type I and II receptors, which phosphorylate SMAD 1, 5, and 9 (human).[9] The phosphorylated regulatory Smads then bind to the co-Smad, SMAD4, and the complex is then translocated into the nucleus for transcriptional regulation of specific target genes such as *ID1–3*, *SNAIL*, and *OASIS*.[9] BMP signaling is regulated by a large network of extracellular antagonists as well as intracellular modulators such as the inhibitory Smads, SMAD6 and 7. SMAD6 is the primary inhibitor of BMP signaling by inhibiting the regulatory Smads and the co-Smad SMAD4 through a negative feedback mechanism (Fig. 1). BMPs play diverse functional roles in neural development and regulation of neural cell progenitors. During development, BMP signaling plays a major role in the patterning and establishment of early embryonic stem cells. During development, the inhibition of BMP signaling is equally critical for neural development. For example, the presence of a BMP gradient created by the presence of both BMP ligands and antagonists establishes the dorsal–ventral axis during development. During embryonic development, neural precursor cells are systematically driven toward specific neuronal or glial differentiation, and BMPs have been shown
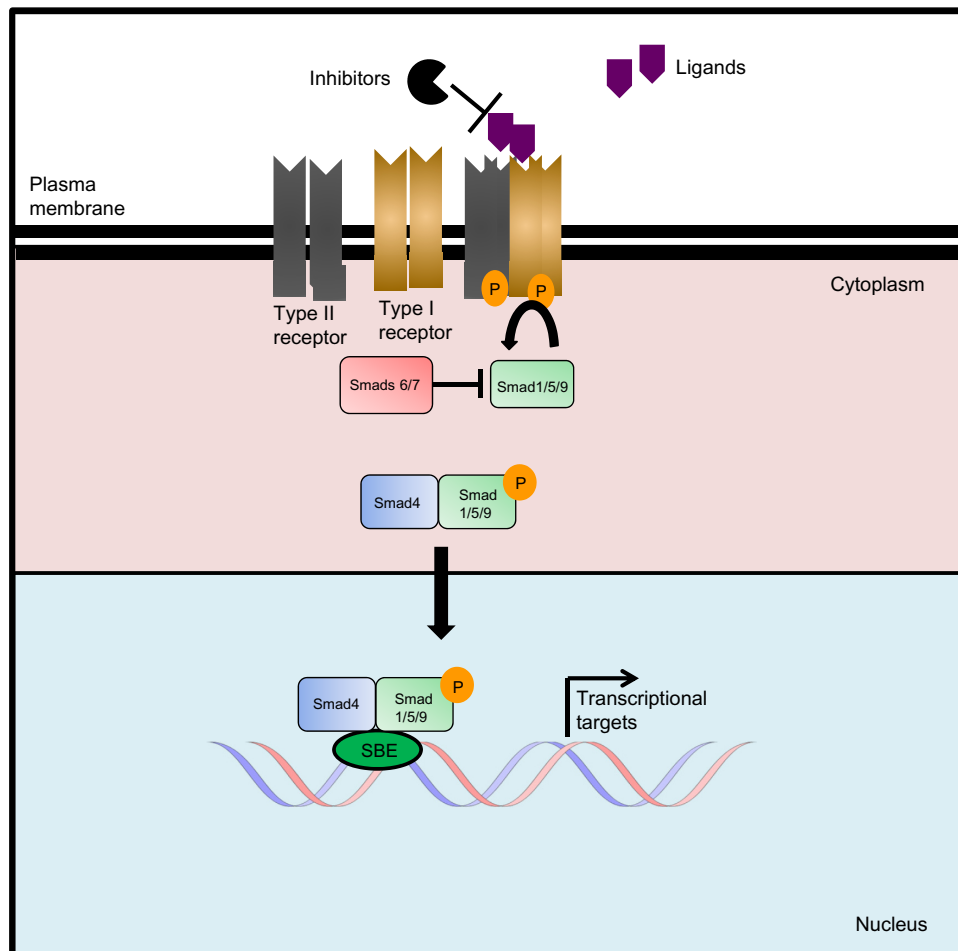
Figure 1. Canonical BMP signaling. The TGFβ family consists of cytokines that signal through serine/threonine kinase receptors. Activated ligands bind to type I and type II serine-threonine kinase receptor complexes, which transduce the signal downstream through the phosphorylation of pathway-specific regulatory Smad proteins (SMADs-1/5/9 are associated with BMP signaling and Smads 2/3 are associated with TGFβ signaling). Generally, the type II receptors phosphorylate the type I receptors, which in turn, phosphorylate the regulatory Smads. Phosphorylated regulatory Smads form a complex with Smad4, the co-Smad, and the complex is translocated to the nucleus for transcriptional regulation. Inhibitory Smads (SMADs 6 and 7) compete with regulatory Smads through both competition for phosphorylation by the type I receptors and binding to Smad4. Signaling antagonists work upstream of the signaling cascade by inhibiting ligands from binding to the receptor complex.

to be a key player in this process by regulating proliferation, mitotic arrest, and apoptosis.[10] Several reviews have been written detailing these effects.[11,12] More recently, it has been discovered that BMP signaling affects the proliferation and differentiation of glioma stem cells (GSC) as well.

BMPs and GSCs. Glioma stem cells (GSCs) are a subpopulation of neoplastic cells within HGGs that have characteristics similar to those of normal neural stem cells. In 2006, Piccirillo et al published a novel finding in which BMP signaling had a striking tumor-suppressive effect on human GBM GSC. Treating human GSC *in vitro* and *in vivo* with BMP4 (a BMP ligand) for 48 hours decreased tumor growth and increased survival in an orthotopic transplant model through a decrease in proliferation and increase in astrocytic differentiation of the GSCs.[13] Subsequently, it has been shown that BMP ligands have a similar impact on both human and murine GSCs.[14–17] Several reviews have been published detailing the major findings in the field discussing BMP-driven tumor suppressive effects on GSCs and initial developments toward clinical BMP therapies for patients.[18,19]

Genomic alteration of BMP-related signaling molecules in human GBM. Although many studies have shown the tumor-suppressive effects of BMP signaling on GSC, little has been reported about BMP signaling in the context of GBM genomics. To gain a better understanding of BMP signaling in human GBMs, we queried BMP pathway alterations at the genetic level to assess how the BMP signaling network is altered in patient samples. We used publically available data compiled and analyzed through TCGA to examine gene expression and mutations and the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) to analyze associations between gene expression and patient survival. We expanded our search beyond the immediate BMP family and analyzed 90 genes within the TGFβ family including receptors, ligands, inhibitors, and downstream targets known to interact directly with the BMP pathway (Supplementary Table 1). To identify genes within the TGFβ family that show either increased or decreased expression in GBMs, we accessed TCGA data using the cBio portal for cancer genomics maintained through the Memorial Sloan Kettering Cancer Center.[20,21] We analyzed the mutational status and mRNA expression of 598 individual GBM samples using the TCGA provisional dataset. mRNA expression is considered to be significantly up or downregulated if the expression is above or below two standard deviations (2σ) of the mean determined from Agilent microarray data. We found that, out of our query of 90 genes, 44 were altered in 5% or more of patients (Table 1). In parallel, we analyzed the association between the mRNA expression of these 90 genes and patient survival using a dataset of 181 GBM patients available using REMBRANDT maintained by the National Cancer Institute (NCI).[22] We investigated whether twofold up or downregulation of the mRNA levels analyzed using the U133 2 Plus mRNA expression chips (Affymetrix) is associated with

increased or decreased overall survival. mRNA increases or decreases in expression are determined in comparison to non-tumor pooled samples.[23] Out of our set of 90 genes, 19 genes were significantly associated with either increased or decreased overall survival (Table 2).

We found four genes, *BMP8B*, *ACVR1B*, *SMAD1*, and *NRTN*, to be both altered in more than 5% of patients and show an association with survival. The role of these four genes in relation to GBMs is largely unknown. *BMP8B* (*OP-2*), first described by Ozkaynak et al in 1992, was found to be a member of the TGFβ family identified through cDNA library screenings. *BMP8B* is expressed early in embryogenesis.[23] Although little is known about this protein in relation to gliomas, BMP8B treatment has been shown to decrease the proliferation of glioma stem cells.[13] Interestingly, the mRNA expression available through the TCGA shows that *BMP8B* mRNA expression is downregulated in 52 patients and upregulated in only 9 patients. Therefore, downregulation of *BMP8B* mRNA expression accounts for 85% of the total *BMP8B* alterations. Within the REMBRANDT GBM dataset, downregulation of *BMP8B* correlates with increased patient survival ($P = 0.02$). The 19 GBM patients within this dataset with downregulated *BMP8B* had an average overall survival of 29.1 months.

ACVR1B (activin A type IB receptor, ALK4) is part of the activin subfamily within the TGFβ family. Activins are members of the TGFβ family known for their role as growth and differentiation factors. *ACVR1B* was originally discovered using a sequence-based polymerase chain reaction (PCR) approach by Dijke et al in 1993. *ACVR1B* mRNA is ubiquitously expressed in all tissues, most strongly in the kidneys, pancreas, brain, lung, and liver.[24] *ACVR1B* mutations have been identified and found to have varying effects in several types of cancer. In prostate cancer, Nomura et al showed that cell lines with constitutively active *AVCR1B* had increased migratory ability aiding in epithelial–mesenchymal transition (EMT). In a neuroblastoma cell line, Suzuki et al showed that *ACVR1B*-specific activin signaling induced neuronal differentiation.[25] Little is known about the role of *ACVR1B* on gliomas. The TCGA data show that *ACVR1B* expression is frequently decreased (41 out of 49 alterations, 85%) when altered in GBMs. Furthermore, within the REMBRANDT dataset, downregulation of *ACVR1B* was associated with decreased survival ($P = 0.0014$). The 82 patients with *ACVR1B* downregulation had an average overall survival of 15.5 months compared to the average overall survival of the 181 patients at 19.6 months.

SMAD1 belongs to the Smad family, a family of proteins that serve as the signal transducers for canonical BMP and TGFβ signaling. While these *Smad* genes were originally discovered and understood in *Drosophila* and *C. elegans*, *SMAD1*, the human homolog, was first discussed and cloned in 1996.[8,26] This protein is activated through BMP receptor phosphorylation, leading to downstream transcriptional regulation. In gliomas, Liu et al showed that phospho-*SMAD1* is expressed

Table 1. Genes altered in ≥5% of human GBMs. All data were collected by the TCGA and analyzed using the cBio Portal for Cancer Genomics. Our dataset consisted of 598 individual GBM samples, and we analyzed 90 genes. For each gene altered in more than 5% of GBMs, we determined in how many tumor samples the gene was mutated and mRNA expression was upregulated or downregulated. mRNA upregulation and downregulation is considered to be >2 standard deviations from the mean expression.

GENES	PERCENT ALTERED IN GBMS (598)	NUMBER OF PATIENTS WITH MUTATED GENE	NUMBER OF PATIENTS WITH mRNA UPREGULATION (>2 STD. DEV)	NUMBER OF PATIENTS WITH mRNA DOWNREGULATION (<2 STD. DEV)
BMP Ligands				
BMP7	5.2	0	15	16
BMP8B	10.2	0	9	52
Receptors				
BMPR1A	5.7	0	1	33
TGFBR1	7.7	2	6	38
TGFBR2	5	2	15	11
ACVR1	6	0	26	10
ACVR1B	8	0	7	41
ACVRL1	7.5	1	13	31
ACVR2B	6	0	29	6
ACVR2A	5	0	26	2
AMHR2	7	1	11	31
Co-receptor				
BMPER	5.5	1	32	0
Intracellular Mediators				
SMAD1	6.5	0	17	22
SMAD2	7.2	0	22	21
SMAD3	6.2	0	13	24
SMAD4	7.7	1	22	23
SMAD6	6.2	0	12	25
SMAD9	7.2	0	12	31
SMURF2	6	0	15	21
IDs				
ID2	6.2	0	20	17
ID3	5.2	0	3	28
Modulators				
CHRD	8.9	1	7	46
AMN	8.7	0	9	43
ENG	6.7	1	14	25
FSTL1	6	1	11	24
SOST	6	0	19	17
TWSG1	5.7	0	10	24
GREM2	5	0	16	14
TGFβ Ligands				
TGFβ1	5	0	14	16
TGFβ2	5	0	12	18
TGFβ3	6.4	0	14	24
Activins				
NODAL	10.7	0	62	2
AMH	8.4	0	8	42
GDNF	7.2	2	12	29

Table 1. (*Continued*)

GENES	PERCENT ALTERED IN GBMS (598)	NUMBER OF PATIENTS WITH MUTATED GENE	NUMBER OF PATIENTS WITH mRNA UPREGULATION (>2 STD. DEV)	NUMBER OF PATIENTS WITH mRNA DOWNREGULATION (<2 STD. DEV)
LEFTY2	5.9	2	16	17
INHBC	5.4	0	16	16
INHBE	5.7	1	26	7
Misc.				
NRTN	9.2	0	11	44
BMP1	7	0	15	27
ARTN	6.4	0	12	26
PSPN	6.2	0	19	18

at lower levels in glioma samples in comparison to normal brain tissue.[27] Additionally, it was found that patients with a high ratio of phosphorylated *SMAD1/5/9* to *SMAD1* had increased survival, demonstrating that increased *SMAD1* activation is beneficial to patient survival.[28] This suggests that increased BMP signaling and increased *SMAD1* phosphorylation provides a survival benefit. Using REMBRANDT, we observed that increased expression of *SMAD1*, found in 65 patients, is associated with decreased survival ($P = 0.02$) when compared to the total overall survival of REMBRANDT GBM patients (15.4 months vs 19.6 months). The mRNA expression data available through the TCGA shows that *SMAD1* mRNA is equally up and downregulated within *SMAD1*-altered GBM samples (17 and 22 patients show upregulation and downregulation, respectively). As described above, analyzing the expression of *SMAD1* in parallel with the phosphorylation of *SMAD1* will be more informative with regard to GBMs and patient survival.

NRTN (neurturin) is a neurotrophic factor serving to promote the survival of various neuronal populations.[27] *NRTN* was first isolated in 1996 by Kotzbauer et al after being identified by its ability to support sympathetic neurons in culture. *NRTN* is closely related to the glial cell line-derived neurotrophic factor (*GDNF*), both known as TGF-beta-related neurotrophins (TRNs). TRNs belong to the TGFβ family based on structural similarity and the presence of conserved cysteine residues found within TGFβ signaling molecules; yet TRNs share less than 20% amino acid sequence similarity to other TGFβ family members.[27,29] Little is known about the role of *NRTN* in gliomas; however it has been reported that *NRTN* promotes pancreatic cell aggressiveness through both proliferation and invasion.[30] The mRNA expression data we examined show that *NRTN* expression is frequently downregulated when altered in GBMs (44 out of 55 alterations). Using the REMBRANDT dataset *NRTN* downregulation is present in 33 patient samples and is associated with decreased survival with the average survival of 13.5 months ($P = 0.01$).

Additional genes of interest. In addition to the genes described above, we chose to further investigate five genes:

SMAD4, BMPR1A, BMP5, ID1, and GREM1. SMAD4, BMPR1A, and *ID1* were primarily selected because of their crucial role in mediating canonical BMP signaling. We selected *BMP5* because we found 4% of all GBM patients possessed upregulation of *BMP5*, which was also found to be associated with increased survival. Finally, *GREM1* was chosen because of the recently published finding showing that glioma stem cells secrete *GREM*1 to promote tumorigenesis through inhibition of BMP signaling.[31]

SMAD4 is a member of the Smad family, and of particular interest, as it is a central regulator of TGFβ family canonical signaling. *SMAD4* was originally discovered as a tumor suppressor in pancreatic cancer in 1996 by Hahn et al after it was found to be homozygously deleted in 25 of 84 pancreatic tumors.[32] *SMAD4* has been shown to be involved in many other types of cancer primarily through chromosome deletion. *SMAD4* has been shown to be inactivated in 48% of pancreatic tumors but is inactivated in less than 10% of tumors in other types of cancer.[33] These deletions have been described in colon cancer,[34] head and neck squamous cell carcinoma,[35] and breast and ovarian cancer.[33] In gliomas, He et al showed that *SMAD4* expression is reduced in all gliomas in comparison with normal brain tissue with the lowest expression in HGGs. In addition He et al found that the loss of *SMAD4* is correlated with poor survival.[36] In our analysis, we found *SMAD4* to be dysregulated in about 8% of the GBM dataset queried with equally distributed up and downregulation (22 and 23, respectively). Furthermore, our analysis showed no significant association between survival and up or downregulation of *SMAD4* mRNA expression ($P = 0.68$, $P = 0.73$ respectively).

BMPR1A (bone morphogenetic protein receptor IA) (also known as *ALK*3), is a type I receptor in the TGFβ family. Using PCR technology, Dijke et al discovered *BMPR1A* in 1993 based on sequence homology to the human activin receptor type II and a type I-like TGFβ receptor in *C. elegans*.[24] Mutations in *BMPR1A* have been shown to cause juvenile polyposis in many patients, a condition characterized by benign growth within the gastrointestinal tract.[37–42] Guo et al in 2014 showed that the micro-RNA, miR-656, acted as a tumor suppressor

Table 2. mRNA expression of genes associated with a significant increase or decrease in overall survival. Using the publically available database REMBRANDT, we were able to determine in which genes upregulation or downregulation of mRNA expression is associated with significant ($P < 0.05$) increased or decreased patient overall survival. Upregulation and downregulation are considered to be more than twofold change from expression in nontumor pooled samples. Log-rank P-values were calculated using the Mantel–Haenszel procedure. For each gene that was associated with overall survival, we determined the number of patients that expressed the alteration and calculated the average overall survival in months. Our dataset consisted of 181 GBM patients with a total overall survival of 19.6 months.

GENE	mRNA EXPRESSION	LOG RANK P-VALUE	ASSOCIATED WITH INCREASED OR DECREASED SURVIVAL:	AVERAGE OVERALL SURVIVAL (MONTHS)	NUMBER OF PATIENTS WITH mRNA ALTERATION
GDNF	Up-Regulated	0.01	Increased	26.3	46
BMP8B	Down-Regulated	0.02	Increased	29.1	19
GDF5	Up-Regulated	0.04	Increased	24.9	36
BMP5	Up-Regulated	0.05	Increased	42.6	7
CHRDL1	Up-Regulated	0.05	Increased	39.2	6
RGMA	Down-Regulated	0.05	Increased	72.9	2
BMP6	Down-Regulated	0.000009	Decreased	8.9	12
GDF15	Down-Regulated	0.000065	Decreased	2.2	1
ACVR1B	Down-Regulated	0.0014	Decreased	15.5	82
GDNF	Down-Regulated	0.01	Decreased	14.0	32
NBL1	Down-Regulated	0.01	Decreased	16.6	110
NRTN	Down-Regulated	0.01	Decreased	13.5	33
SMAD6	Up-Regulated	0.01	Decreased	10.4	10
SOST	Up-Regulated	0.01	Decreased	14.0	45
TSKU	Up-Regulated	0.01	Decreased	17.8	152
FSTL1	Up-Regulated	0.02	Decreased	18.1	154
INHBE	Down-Regulated	0.02	Decreased	14.7	39
SMAD1	Up-Regulated	0.02	Decreased	15.4	65
TWSG1	Up-Regulated	0.02	Decreased	17.5	121

in gliomas by specifically repressing expression of *BMPR1A*.[43] Our analysis from TCGA data shows that *BMPR1A* mRNA expression is altered in approximately 5.7% of GBMs, with the vast majority of those being downregulated (33 out of 34 alterations). Within the REMBRANDT dataset, there were no GBM patient samples available with downregulation of *BMPR1A*. We found no association between upregulation of mRNA expression of *BMPR1A* and survival ($P = 0.23$).

BMP5 (Bone morphogenetic protein 5) BMP ligands were originally identified by Urist in 1965 because of their ability to induce endochondral osteogenesis *in vivo* at an extraskeletal site.[44] *BMP5*, based on sequence homology, is in a subgroup with BMP 6, 7, and 8b.[45] In adrenocortical carcinoma and pancreatic cancer, expression of *BMP5* was found to be downregulated.[46,47] *BMP5* was shown to inhibit cell proliferation yet increase migration and invasion in pancreatic cancer cell lines.[47] *BMP5* has been shown to decrease proliferation of glioma stem cells.[13] Our mRNA expression data show that *BMP5* is altered in 4.8% of GBMs. Eighty-six percent of those alterations were due to upregulation of *BMP5* (25 out of 29 alterations), and we found upregulation of *BMP5* to be associated with increased survival of GBM patients ($P = 0.05$). The seven patients available within the

REMBRANDT database with upregulation of *BMP5* had an average overall survival of 42.6 months, more than twice the average survival.

ID1 (inhibitor of DNA-binding 1) is a key transcriptional regulator that is a specific downstream target of active BMP signaling. ID proteins inhibit the binding of DNA to other transcriptional factors by binding to the helix–loop–helix motif of transcriptional factors. *ID1* was isolated from human fibroblasts in 1994 by Hara et al.[48] *ID1* has been shown to regulate the cell cycle and differentiation of cells in a wide variety of cell types[48–51] including normal neural and glioma cells. In 2009 Nam and Benezra showed that *ID1* and *ID3* are required to maintain self-renewal of the type B adult neural stem cells. In addition, it was shown that *ID1* can be used to identify type B neural stem cells within the stem cell niches of the brain and that ID1 protein levels decrease during the process of cell differentiation.[52] However, the role of *ID1* in GBM biology has proven to be very complex. *ID1* expression has been shown to be upregulated in human gliomas and murine experimental models of glioma.[53,54] In 2013, Soroceanu et al showed that *ID1* levels correlate with tumor histopathologic grades and tumor cell invasiveness *in vitro* and that knock-down of *ID1* increased survival in an orthotopic model of

GBM.[55] Contrastingly, Barrett et al showed, using a murine model of HGG, that glioma cells with both high and low levels of *ID1* are tumorigenic and, surprisingly, the low *ID1* expressing cells formed tumors more rapidly and with higher penetrance.[56] According to our analyses, *ID1* expression is altered in approximately 4.5% of GBMs, equally up and down-regulated in the TCGA sample population (13 patients each). In the patient samples available through REMBRANDT, the five GBM patients with low *ID1* mRNA expression had significantly decreased survival with an average overall survival of 8.5 months ($P = 0.01$). *ID1* plays a prominent role in regulating both normal and tumor cells and warrants further investigation.

GREM1 (Gremlin 1) is an antagonist of BMP signaling. GREM1 is a secreted molecule that binds to BMP ligands to prevent them from binding to their receptors. *GREM1* was isolated in 1998 by Hsu et al by cloning the human homolog of the gremlin gene in Xenopus.[57] *GREM1* is a crucial mediator of development by restricting BMP signaling.[58] We chose to specifically investigate the expression of *GREM1* due to the recent publication: "Glioma cancer stem cells secrete Gremlin1 to promote their maintenance within the tumor hierarchy" by Yan et al in 2014. This publication shows that *GREM1* is endogenously expressed by GSCs to protect their self-renewal ability and stem-like state from the pro-differentiation effects of BMP signaling. GREM1 secretion is thought to contribute to treatment resistance through maintaining cellular proliferation and cellular hierarchies within the tumor, as well as increasing resistance to differentiation therapy.[31] Our analysis shows that *GREM1* is altered in approximately 3.8% of GBMs (23 patients), all of which are mRNA upregulations. Interestingly, using the REMBRANDT dataset, mRNA upregulation was present in 12 of the 181 patients showing enhanced survival with an average survival of 40 months, which is more than twice the average survival ($P = 0.06$).

Conclusions and Future Outlook

The BMP signaling pathway is a complex network of receptors, ligands, and antagonists, all of which may be capable of dynamically impacting GBM growth, maintenance, and progression, both positively and negatively. As with TGFβ signaling, it appears as though BMP signaling can modulate tumor growth and maintenance in various ways and most likely plays a context-dependent role in GBM tumor growth. Our analysis shows that upregulation and downregulation of ligands, receptors, and intracellular modulators are associated with both increased and decreased survival. Similarly, previous reports have shown conflicting data regarding the expression of various BMP signaling molecules and survival in human GBM.[59–61] To better understand this pathway and how we may be able to exploit the signaling for novel drug treatments, we examined patient samples at the genetic level to explore the BMP pathway in GBMs. BMP signaling is required for development and the regulation of neural cells. As reviewed

by Guang-Quan Zhao et al in 2002, targeted mutagenesis of BMP ligands, receptors, and other pathway modulators has shown that BMP signaling is involved and critical to almost all aspects of development.[62] Given the high importance of BMP signaling, especially with a focus on neural cells, we hypothesize that BMP signaling is likely necessary for the survival of tumor cells and is very tightly regulated within the tumor environment, which may explain why the pathway is attenuated and not genetically deleted within GBMs. In support of our hypothesis, we observed that within the 598 tumors surveyed using the TCGA GBM provisional dataset, none of the genes queried are altered in more than 15% of the tumors, and mutations are exceedingly rare. The majority of the genes examined are not altered in more than 5% of GBMs. The alterations seen primarily occur in nonoverlapping tumors, indicating that compensation or redundancy in the pathway is possible and perhaps necessary for survival of tumor cells in GBMs. However, we may be able to exploit this importance to tumor cell survival in a way to benefit patients. As shown in xenograft models, there have been several forms of BMP treatments that have shown increased survival and decreased tumor growth, which should be considered for development in the clinic.[13,17,63,64] More studies need to be completed to show that increased levels of BMP signaling *in vivo* at high doses do not act as tumor promoters. Understanding the larger impact of BMP treatment on the bulk of the tumor and the microenvironment is crucial prior to the development of BMPs into the clinic. As our analysis shows, increased expression of molecules involved in BMP signaling is not always associated with increased survival. The nature of genomic studies allows for a network view of individual pathways and uncovers genes within the pathway that may have remained unnoticed. Our analysis primarily serves as a guide for future research, emphasizing genes that are altered in a significant number of patients and are associated with survival. Here we highlighted several genes largely unstudied in the context of GBMs, which are both altered in a high percentage of patients and have associations with survival. Our analysis of 90 genes related to the BMP pathway provides a network view of the alterations occurring within the pathway to complement the many single-gene research studies that have been done. However, there are several factors that should be considered when interpreting this data. GBMs are known for their intratumoral heterogeneity; therefore it is likely that the gene expression and gene alterations differ throughout the tumor. Additionally, it is impossible to know when these mutations and alterations arose during the progression of the tumor. It is unknown which of the alterations reported were present in the initial tumor-promoting cells and which have been acquired in response to the selective pressures of the tumor microenvironment, treatments, and tumor resections. Johnson et al demonstrated this phenomena showing vast differences in the genomic alterations and mutations present in initial and recurrent tumors.[32] For example, Johnson et al showed that

SMADs 4,6,7, and *9* were mutated in recurrent tumors and not initial tumors, suggesting that alterations in Smads may not be involved in driving the initial tumor formation.[32] Finally, our analysis is based on limited patient data with diverse genetic and treatment variables, which are important components to consider when determining appropriate therapies.

Given these caveats, our ultimate goal in sharing these findings is that our analysis will guide future studies to a novel and more complete understanding of the BMP pathway in relation to GBM pathology. As more genomic information is acquired, and as diverse samples are rapidly added to these publically available datasets, we will be able to generate more distinct and conclusive data on the genetic alterations and mutations that are critical regulators in GBM development and progression. In future studies with the addition of patient data, we will be able to stratify the population based on factors such as tumor subtype, initial or recurrent tumors, or previous treatments to take a more hypothesis-driven approach to genomic analysis. Recently, GBM genomic studies have begun to explore the extent of intratumoral heterogeneity when stratified by tumor stage or at the single-cell level enforcing the need for larger, increased depth of publically available GBM datasets.[32,65]

As we begin to combine the rapidly growing knowledge of epigenetic and proteomic information with genomic studies, our understanding of GBM tumor biology will vastly increase. Our current understanding of these tumors is clearly not proficient, given the dismal, almost uniformly fatal, outcome of this disease; however, genomic analyses can lead to new diagnostic tests, classifications, and treatment combinations and will direct both basic scientists and clinicians toward a future of successful individualized treatments. With the arrival of personalized medicine, it is more imperative than ever to gain a further understanding of these heterogeneous tumors at the genetic level for the optimization of new therapies.

Materials and Methods

TCGA analysis: cBioPortal for cancer genomics. Gene mutation status and mRNA expression were analyzed using publically available data obtained through the cBio Cancer Genomics Portal ((http://www.cbioportal.org/public-portal/ (accessed May 2014)). We selected the GBM (TCGA, Provisional) dataset from the Brain CNS Cancer Study category. Within the Genomic Profiles options, we selected mutations and mRNA expression data from Agilent microarray data using a z-score threshold of 2.0. Z-scores were determined by comparing the mRNA expression of each tumor sample to the mean expression value of all tumors that are diploid for the gene of interest. At the time of access, there were 598 patients available within the "All Tumors" GBM TCGA provisional dataset.

Survival analysis: REMBRANDT. Microarray gene expression and survival data were acquired from the publically available NCI REMBRANDT database (https://caintegrator. nci.nih.gov/rembrandt/home.do (accessed May 2014)). To analyze associations with survival, we selected the graph format: Kaplan-Meier survival plot for Gene Expression Data. We restricted the analysis to GBM patient samples. At the time of access, there were 181 GBM samples. Gene expression was determined from U133 2 Plus mRNA expression chips (Affymetrix). Upregulation and downregulation were determined as twofold or greater difference than pooled nontumor samples. Log-rank *P*-values were calculated using the Mantel–Haenszel procedure to determine the significance between groups of samples stratified by levels of gene expression.[66] Gene associations with overall survival were compared to the overall survival for all 181 GBM patients. The average overall survival for all 181 patients was 19.6 months. To determine the average overall survival, we reviewed the clinical reports of patients segregated by gene expression and calculated the mean overall survival.

Author Contributions

Conceived and designed the experiments: LDH, PO. Analyzed the data: LDH, PO. Wrote the first draft of the manuscript: LDH. Contributed to the writing of the manuscript: LDH, TWA, PO. Agree with the manuscript results and conclusions: LDH, TWA, PO. Jointly developed the structure and arguments for the paper: LDH, TWA, PO. Made critical revisions and approved final version: LDH, TWA, PO. All authors reviewed and approved of the final manuscript.

Supplementary Data

Supplementary Table 1. List of 90 genes related to TGFβ family that was analyzed at the genomic level. Genes were analyzed at the mRNA level for both alterations and any associations with survival in GBM patients.

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