CSNK1A1 and Gli2 as Novel Targets Identified Through an Integrative Analysis of Gene Expression Data, Protein-Protein Interaction and Pathways Networks in Glioblastoma Tumors: Can These Two Be Antagonistic Proteins?

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ABSTRACT: Glioblastoma (GBM) is the malignant form of glioma, and the interplay of different pathways working in concert in GBM development and progression needs to be fully understood. Wnt signaling and sonic hedgehog (SHH) signaling pathways, having basic similarities, are among the major pathways aberrantly activated in GBM, and hence, need to be targeted. It becomes imperative, therefore, to explore the functioning of these pathways in context of each other in GBM. An integrative approach may help provide new biological insights, as well as solve the problem of identifying common drug targets for simultaneous targeting of these pathways. The beauty of this approach is that it can recapitulate several known facts, as well as decipher new emerging patterns, identifying those targets that could be missed when relying on one type of data at a time. This approach can be easily extended to other systems to discover key patterns in the functioning of signaling molecules. Studies were designed to assess the relationship between significant differential expression of genes of the Wnt (Wnt/β-catenin canonical and Wnt non-canonical) and SHH signaling pathways and their connectivity patterns in interaction and signaling networks. Further, the aim was to decipher underlying mechanistic patterns that may be involved in a more specific way and to generate a ranked list of genes that can be used as markers or drug targets. These studies predict that Wnt pathway plays a relatively more pro-active role than the SHH pathway in GBM. Further, CTNNB1, CSNK1A1, and Gli2 proteins may act as key drug targets common to these pathways. While CTNNB1 is a widely studied molecule in the context of GBM, the likely roles of CSNK1A1 and Gli2 are found to be relatively novel. It is surmised that Gli2 may be antagonistic to CSNK1A1, preventing the phosphorylation of CTNNB1 and SMO proteins in Wnt and SHH signaling pathway, respectively, by CSNK1A1, and thereby, aberrant activation. New insights into the possible behavior of these pathway molecules relative to each other in GBM reveal some key interesting patterns.

KEYWORDS: glioblastoma, Wnt and SHH signaling pathways, integrative analysis, gene expression, protein–protein interaction networks, bottleneck nodes, simultaneous targeting, β-catenin (CTNNB1), casein kinase 1, alpha 1 (CSNK1A1) and glioma-associated oncogene 2 (Gli2) proteins

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Introduction

Glioblastoma (GBM) is the most malignant of all the brain tumors with very low median survival time of one year, as per Central Brain Tumor Registry of the United States, 2001. Several groups have initiated high-throughput molecular profiling studies toward a better understanding of GBM.[1–5] GBMs, also known as grade IV gliomas, are a heterogeneous bunch of tumors arising from astroglial cells and sometimes from oligodendrocytes and are characterized by four distinct molecular subtypes on the basis of gene expression, copy number changes, and DNA sequence alterations, *viz.*, neural, proneural, mesenchymal, and classical subtypes.[5] The neural subtype is defined by the presence of neuron markers such as NEFL and SLC12A5, whereas the proneural subtype is characterized by the expression of proneural development genes such as *SOX, DLL3, OLIG2*, and *TCF4*, as well as high levels of expression of PDGFRA and p53 mutations. Mesenchymal subtype is characterized by high-level expression

of genes in NF-κB pathway, as well as tumor necrosis factor (TNF) superfamily pathway, with mutations in *NF1* and *PTEN* tumor suppressor genes. High-level EGFR amplification with high-level expression of genes of Notch pathway, sonic hedgehog (SHH) pathway, and *NES* gene, and absence of p53 mutations define the classical subtype of GBM.

Among the major pathways studied in GBM tumors, aberrant activation of Wnt/β-catenin signaling pathway, as well as SHH signaling pathway has been reported.[6,7] The aberrant activation of these pathways is one of the many mechanisms that lead to cellular migration, proliferation, and enhanced survival of tumor cells. Further, these two pathways are also involved in the maintenance, proliferation, and clonogenicity of glioma cancer stem cells.[8] These cancer stem cells have a role to play in the initiation, proliferation, and invasion in gliomas, and therefore, can be one of the several important points of therapeutic intervention.

In normal cells, these pathways are involved in vertebrate organogenesis, morphogenesis, and other developmental roles. Several similarities between these pathways during their signal transduction events can be identified[9] such as activation through a G-protein-coupled receptors (GPCRs)-related membrane protein and prevention of phosphorylation-dependent proteolysis of β-catenin (CTNNB1) effector. This effector molecule helps activate target genes through conversion of a repressor protein (TCF) into an activator protein. Several other roads and milestones in these two pathways are pathway specific.[9]

Studies have found an overexpression of Wnt ligands of canonical pathway, Wnt1 and Wnt3a, in high-grade gliomas.[10] Non-canonical Wnt signaling pathway ligand, Wnt5a, was also found to be involved in tumor progression.[11] Another study observed an overexpression of Wnt5a and Wnt7b, as well as Frizzled proteins Fzd-2, -6, and -7 in glioma cells.[12] In the case of SHH pathway, expression of SHH pathway genes such as *PTCH, SMO, Gli1*, and *Gli2* was observed in CD133-positive malignant glioma cells, and this pathway was found to be playing an important role in cellular migration of these cells.[13]

Keeping in view the similarities as well as the differences between these pathways and their likely co-ordinated role in GBM tumor progression, there arises a need to explore their contextual functioning in more detail, particularly the genes' behavior in relation to each other. Further, it will be useful to discern a specific molecule or set of molecules common to these pathways that can serve as potential drug target/s so that these pathways can be targeted simultaneously. These drug targets can, more often, be "bottlenecks" in a pathway,[14] ie, the bottleneck genes/gene products which connect two or more pathways together and therefore are more likely, essential genes/gene products. One of the approaches may, thus, involve cohesive integration of both gene expression data and different types of networks involving these genes or their products. Using this approach, gene/s with a potential as attractive drug target candidates that are usually overlooked when relying on differential gene expression analysis or protein–protein interaction (PPI) networks alone can be enumerated as a ranked list. This approach will also be useful in providing more insights into their behavior in context.

Materials and Methods

Dataset assembly. From The Cancer Genome Atlas (TCGA) website, level 3 normalized and processed gene expression dataset for 49 genes coding for ligands, receptors, co-receptors, destruction complex, transcriptional effectors, antagonists, downstream targets, tumor suppressors, and apoptotic genes involved in SHH, as well as Wnt/β-catenin canonical and non-canonical Wnt signaling pathways (Table 1) was compiled. In all, data belonging to a total of 431 GBM and 10 normal tissue samples were downloaded. The microarray platform used was Affymetrix HT_HG-U133A platform and the GBM samples were primary GBM samples.

Significant differential gene expression analysis. Significant differential gene expression was analyzed using both the significance analysis of microarrays (SAM) and *T*-test modules of MultiExperiment Viewer (MeV) version 4.6. Two different statistical tests were used in order to enhance confidence in predictions of significantly differentially expressed genes. A default *p*-value cutoff of 0.01 was used to assess significant differential expression using *T*-test. Differential gene expression was considered significant if false discovery rate was <0.05 and delta-value was 1.0 using 1000 permutations in SAM, and this cutoff was used in order to enlist a majority of significantly differentially expressed genes, as well as biologically meaningful relationships. Comparative marker selection analysis with default parameters from GenePattern suite of tools was used to assess upregulation or downregulation of these genes.

Network assembly. In order to gain a comprehensive understanding, several types of networks such as PPI, co-expression, co-localization networks, and pathways were constructed using GeneMania plugin installed in Cytoscape version 3.0. GeneMania uses several quality data sources to assemble validated networks such as GEO for co-expression network, BioGrid for physical interaction (PPI) networks, PathwayCommons for pathways network, among others. In brief, the interaction/association dataset for the organism *Homo sapiens* was installed locally from GeneMania plugin and in combination with Cytoscape 3.0, used for the assembly of a new network for further studies. In all, this installed dataset comprised 144 networks with 21,438 genes (nodes) and thousands of interactions (edges) among these genes. From this dataset, new networks were assembled for all the genes or gene products included in the dataset under study (Table 1). Some neighboring genes, which were not part of dataset in Table 1, were added automatically by GeneMania plugin, with the top 20 related

Table 1. Wnt and SHH signaling pathway genes used in this study categorized as ligands, receptors, co-receptors, destruction complex, transcriptional effectors, antagonists, downstream targets, tumor suppressors, and apoptotic genes.

WNT PATHWAY:			
PATHWAY COMPONENTS	ENTREZ GENE ID	GENE SYMBOL	GENE NAMES
Ligands	7471	WNT1	Wingless-Int1
	7474	WNT5 A	Wingless-Int5A
	7482	WNT2B	Wingless-Int2B
Receptors	2535	FZD2	Frizzled2
	7855	FZD5	Frizzled5
	7976	FZD3	Frizzled3
	8321	FZD1	Frizzled1
	8322	FZD4	Frizzled4
	8323	FZD6	Frizzled6
	8324	FZD7	Frizzled7
	8325	FZD8	Frizzled8
	8326	FZD9	Frizzled9
	11211	FZD10	Frizzled10
Co-receptors	4040	LRP6	Low Density Lipoprotein Receptor-related Proteins-6
	4041	LRP5	Low Density Lipoprotein Receptor-related Proteins-5
Transcriptional Activators	1499	CTNNB1	Beta-Catenin
β-catenin	2932	GSK3β	Glycogen synthase kinase 3 β
destruction	324	APC	Adenomatous polyposis coli
complex	8312	AXIN1	Axin
	1452	CSNK1A1	Casein kinase 1, alpha 1
Effectors	6932	TCF7	Transcription factor 7 (T cell specific, HMG box)
	6934	TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
	83439	TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)
	51176	LEF1	Lymphoid enhancer-binding factor 1
	1855	DVL1	Dishevelled-1
	1856	DVL2	Dishevelled-2
	1857	DVL3	Dishevelled-3
Canonical pathway activators	10023	FRAT1	Frequently rearranged in advanced T-cell lymphomas-1
	23401	FRAT2	Frequently rearranged in advanced T-cell lymphomas-2
Wnt antagonists	22943	DKK1	Dickkopf1
	6422	SFRP1	Secreted Frizzled-related protein 1
Downstream targets	595	CCND1	Cyclin D1
	652	BMP4	Bone morphogenetic protein 4
	891	CCNB1	Cyclin B1
	894	CCND2	Cyclin D2
	999	CDH1	E-cadherin
	4609	c-MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
SHH PATHWAY:			
PATHWAY COMPONENTS	ENTREZ GENE ID	GENE SYMBOL	GENE NAMES
Ligand	6469	SHH	Sonic Hedgehog
Receptors	5727	PTCH1	Patched-1
	8643	PTCH2	Patched-2

(*Continued*)

Table 1. (*Continued*)

SHH PATHWAY:			
PATHWAY COMPONENTS	**ENTREZ GENE ID**	**GENE SYMBOL**	**GENE NAMES**
Transcriptional Activators	2735	GLI1	GLI Family Zinc Finger 1
	2736	GLI2	GLI Family Zinc Finger 2
Destruction complex	2932	GSK3β	Glycogen synthase kinase 3 β
	1452	CSNK1A1	Casein kinase 1, alpha 1
Effectors/Downstream targets	2735	GLI1	GLI Family Zinc Finger 1
	2736	GLI2	GLI Family Zinc Finger 2
	2737	GLI3	GLI Family Zinc Finger 3
	6608	SMO	Smoothened
	595	CCND1	Cyclin D1
	652	BMP4	Bone morphogenetic protein 4
	891	CCNB1	Cyclin B1
	894	CCND2	Cyclin D2
	3714	JAG2	Jagged 2
	4609	c-MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
	6422	SFRP1	Secreted Frizzled-related protein 1
Apoptotic gene	355	FAS	Fas Cell Surface Death Receptor
PATHWAY COMPONENTS	**ENTREZ GENE ID**	**GENE SYMBOL**	**GENE NAMES**
Tumor suppressors	999	CDH1	E-cadherin
	5728	PTEN	Phosphatase And Tensin Homolog
	6598	SMARCB1	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin Subfamily B Member 1
	6615	SNAI1	Snail Family Zinc Finger 1

genes chosen to be added to the network with automatic weighting (default) option.

Network analysis. Network analysis for node degree, color-coded expression values, and betweenness centrality, among others, was done using Network Analyzer plugin, VistaClara, and VizMapper in Cytoscape 2.8. First neighbors of selected nodes to determine directly connected nodes were analyzed using Select menu of Cytoscape 2.8.

Node degree in a network denotes number of edges/connections in an undirected interaction network, ie, how much connected a node is to all other nodes in the network. More densely connected nodes have a higher node degree and are considered as "hub" molecules. The gene expression values, when superimposed on a network, provide a way to visualize network in terms of nodes corresponding to significantly differentially expressed genes. A green–red color gradient denotes lower–higher expression values for gene products of significantly differentially expressed genes, and green-yellow-red color gradient denotes nodes with lower to higher node degree. VistaClara plugin was used to superimpose the gene expression values on the network, and VizMapper was used to generate the color gradient. Betweenness is an important

topological property of a network that defines the number of shortest paths that are non-redundant going through a particular node. Since these nodes tend to be critical points, these can be thought of as bottleneck nodes without which the information flow would be virtually impossible. Higher the betweenness, more essential and critical the molecule is likely to be. Depending upon "hubness" (node degree) and "betweenness," the bottleneck nodes are classified as (a) hub–non-bottlenecks; (b) non-hub–non-bottlenecks; (c) non-hub–bottlenecks; and (d) hub–bottlenecks. The nodes in the network have been colored using a green-red color gradient for assessing their lower–higher betweenness centrality, using Network Analyzer to calculate the betweenness centrality and VizMapper to color the nodes according to this measure.

Results and Discussion

Majority of genes encoding ligands, receptors, co-receptors, regulators, and transcriptional effectors among others involved in SHH, as well as Wnt/β-catenin canonical and Wnt non-canonical signaling pathways are upregulated and significantly differentially expressed in GBM. Wnt/β-catenin and SHH pathway genes are aberrantly

activated in GBM. Upregulation of some of these pathway genes has been reported in literature as mentioned earlier. Genes in these signaling pathways functioning as ligands, receptors, co-receptors, destruction complex, transcriptional effectors, antagonists, downstream targets, tumor suppressors, and apoptotic genes (Table 1) were studied for their expression and interaction patterns. In all, a total of 49 genes were analyzed, and on the basis of comparative marker selection analysis results, 28 genes were found to be upregulated and 9 genes downregulated in GBM (Table 2). SAM and T-test analyses both pointed to a majority of genes being significantly differentially expressed. Out of a total of 37 significantly differentially expressed genes that were enlisted using SAM and T-tests, 33 genes were observed to be significantly differentially expressed by both these tests, and three genes were found to be so by either of these. The significant differential expression is analyzed in the context of both tumor and normal tissues. Their respective q-values in percent, which is the likelihood of a false positive case, at FDR value set at <0.05 or <5% and p-values set at 0.01, are given in Table 2. It is seen from this table that q-values and p-values for all of the genes listed, except one, fall within the given cutoff. Some genes with significant differential expression may be upregulated in tumors and some may be upregulated in normal tissues (downregulated in tumors), as detailed below.

Significant differential expression of members of SHH signaling pathways. Genes such as *CSNK1A1, PTCH2, GSK3β,* and *Gli2* were found to be significantly differentially expressed, whereas *SHH* as well as *Gli1, Gli3,* and *PTCH1* genes were not significantly differentially expressed. Of these, *CSNK1A1* and *Gli2* were found to be upregulated in tumors. Low-level expression of SHH ligand in tumors is unexpected since it may be needed for the SHH signaling pathway to proceed. However, several studies have also reported a low-level expression of SHH in tumors.[15,16] Braun et al.[15] found in their studies that there was no correlation between Hh activity and the levels of *SHH, Gli1,* and *PTCH1* mRNA expression in tumor cells derived from GBM and that there was very low overall expression of SHH. Bar et al.[16] reported SHH activity in some, as opposed to all, primary GBM tumors and speculated that "the SHH mRNA we detected in primary glioma samples was being generated by non-neoplastic cells and that pure tumor cultures are therefore negative." Ehtesham et al.[17] also mention similar results that SHH pathway is activated in Grade II and III gliomas, but not in Grade IV *de novo* GBM tumors. Taken together, this may be interpreted to mean that the Hh pathway in GBM may progress *via* a ligand other than SHH or in a ligand-independent manner. Further, ligand-independent function may occur due to loss-of-function mutation in PTCH or gain-of-function mutation in SMO, as mentioned in several studies.

Verhaak et al.[5] using TCGA dataset in their analyses mentioned that "Sonic hedgehog (*SMO, GAS1, GLI2*) signaling pathways were highly expressed in the Classical subtype," similar to studies in this current paper. Interestingly, there was no mention of SHH ligand expression in the paper by Verhaak et al.

Table 2. Significantly differentially expressed genes upregulated in tumors, false discovery rate or *q*-value <0.05 or <5% (likelihood of a false positive case), and delta-value 1.0 were used in SAM analyses and *p*-value cutoff of 0.01 was used for *T*-test.

S. NO.	GENES	Q-VALUE(%)	P-VALUE
1.	WNT5A	0.0	0.0
2.	CSNK1A1	0.0	0.0
3.	FZD7	0.0	7.79E-14
4.	FZD6	0.0	0
5.	CCNB1	0.0	5.48E-10
6.	LRP5	0.0	0.0
7.	FZD1	0.0	5.46E-10
8.	TCF7L1	0.0	1.71E-07
9.	c-MYC	0.0	1.73E-06
10.	FZD2	0.0	1.61E-06
11.	FAS	0.0	2.27E-05
12.	DVL3	0.0	1.38E-06
13.	DVL2	0.0	1.32E-05
14.	CTNNB1	0.0	9.83E-06
15.	LEF1	0.0	1.57E-05
16.	CCND1	0.0	1.46E-05
17.	TCF7L2	0.0	5.02E-06
18.	DKK1	0.9	7.18E-04
19.	FZD5	0.0	3.50E-05
20.	SMARCB1	0.0	0.001261
21.	GLI2	3.4	4.03E-05
22.	TCF7	3.4	2.18E-04
23.	LRP6	0.0	4.94E-07
24.	FZD4	3.4	5.31E-05
25.	FZD10	0.0	1.87E-05
26.	AXIN1	1.0	*
27.	SMO	NaN**	*
28.	CDH1	NaN	9.22E-04

Significantly differentially expressed genes upregulated in normal tissue samples, false discovery rate or *q*-value <0.05 or <5% (likelihood of a false positive case) and delta-value 1.0 were used in SAM analyses and *p*-value cutoff of 0.01 was used for *T*-test.

S. NO.	GENES	Q-VALUE(%)	P-VALUE
1.	WNT1	0.95	*
2.	FZD9	0.0	0.004177
3.	GSK3β	0.0	0.005612
4.	SFRP1	1.0	0.001744
5.	PTCH2	0.0	0.001241
6.	WNT2B	0.0	5.56E-05
7.	DVL1	0.0	1.06E-05
8.	JAG2	0.0	8.05E-06
9.	APC	0.0	5.15E-12

Notes: *Not significant. **Differential expression in Figure 1. NaN: *q*-value not calculated.

Significant differential expression of members of Wnt signaling pathways and other genes implicated in the signaling process. Majority of members of Wnt signaling pathways were significantly differentially expressed, as well as upregulated in tumors in contrast to relatively few members of SHH signaling pathway. This shows that in comparison to SHH signaling, Wnt signaling mechanisms are more pro-active in GBM tumors. In brief, significantly differentially expressed genes such as *CTNNB1, CSNK1A1, Frizzled receptors, LRP5, LRP6, TCF7L1, TCF7L2,* and *LEF1,* among others, were upregulated in tumors. Among significantly differentially expressed Wnt ligands, non-canonical signaling molecule, Wnt5a, was found to be upregulated and canonical signaling molecules such as Wnt1 and Wnt2b downregulated in tumors. In fact, significant differential expression was highest in the case of two molecules: Wnt5a and CCNB1. These results are consistent with another study,[18] where non-canonical Wnt signaling molecule Wnt5a was found upregulated in GBM, whereas canonical Wnt signaling molecules such as Wnt1 were not regulated as compared to normal brain. CCNB1 is known to contribute to cellular proliferation, lending it an important role in GBM progression. The non-canonical Wnt5a signaling pathway is a CTNNB1-independent pathway, but may also activate Wnt/CTNNB1 canonical signaling in the presence of Fzd4 and LRP5.[19] The fact that Fzd4 and LRP5 are significantly differentially expressed as well as upregulated in tumors along with Wnt5a in the current study lends credence to the theory that Wnt5a may be activating the canonical pathway in GBM as well.

Other significantly differentially expressed genes found to be upregulated in tumors were *SMARCB1* and *FAS* cell surface death receptor genes. This is interesting given the fact that *SMARCB1* acts as a tumor suppressor gene in malignant rhabdoid tumors, and given its function, should be downregulated in tumors, but its role in GBM is not fully studied. However, many tumor suppressor genes such as *p16INK4a* have been found to be overexpressed in a wide variety of tumors[20] and may provide evidence, in part, that the upregulation of *SMARCB1* in GBM observed in the current study may be related to GBM development, and therefore, needs further exploration. It is surmised that the upregulation of *FAS* cell surface death receptor gene, which leads to apoptosis, is circumvented, in part, by the upregulation of Wnt signaling proteins, mainly by Wnt5A, which has been shown to drive apoptosis resistance in pancreatic cancer cells.[21] SHH signaling may also play a role.[22]

SFRP1, JAG2, GSK3β, and *APC* genes were found significantly upregulated in normal tissues. *SFRP1* is a putative tumor suppressor gene and an antagonist of Wnt non-canonical signaling and JAG2 is a Notch ligand, both proteins being HH signaling targets. Their significant differential upregulation in normal tissue samples provides further evidence that hedgehog pathway is less active than Wnt pathway in GBM. DKK1, an antagonist of Wnt canonical

signaling pathway, is upregulated in tumors and may inhibit this pathway, although Wnt5a molecule may serve to overcome this activity as has been explained above. GSK3β and APC are parts of CTNNB1 destruction complex, their downregulation in tumor cells may lead to loss of activity of destruction complex and hence, stabilization of CTNNB1, which functions as transcriptional co-activator of TCF/LEF family of transcription factors.

CSNK1A1 and Gli2 are the novel targets identified through an integration of gene expression data and network connectivity patterns. Several groups have used PPI networks to understand the patterns of connectivity between genes or gene products. Information on key gene/s or gene product/s acting as "hub" molecules with a high degree of connectivity, and which are distinct from their neighboring genes in gene expression patterns, can be used to leverage their potential as attractive drug target/s. To identify key gene products common to both pathways that can be targeted simultaneously and to minimize the chances of important genes being overlooked when relying on single type of analyses, significant differential gene expression analyses and network connectivity patterns were integrated together.

PPI network. PPI networks were overlaid with gene expression data to further generate an underlying pattern (Fig. 1A and B). It was observed that CTNNB1 and CSNK1A1 were the top two genes/gene products that were both significantly differentially expressed and highly connected with the number of nodes (gene/gene product) being 40 and 26, respectively. It was further seen that for most of the other gene products, the number of connections to other nodes, ie, node degree was not related to the expression level. As an example, Wnt5a, which has highest level of significant differential expression and is upregulated in tumors, has lower node degree as compared to LRP6, which has comparatively lower level of expression but a higher node degree (Fig. 1C).

Further, the proteins encoded by genes that are not significantly differentially expressed, showed some degree of connectivity to those proteins that were encoded by significantly differentially expressed genes. As an example, Fzd8, which was not significantly differentially expressed was found connected to LRP5, LRP6, and Wnt1, all of which are significantly differentially expressed, each with its own interacting partners encoded by significantly differentially expressed genes. This suggests that Fzd8 may be playing an important role, in spite of its differential expression not being significant.

Pathway network. In the pathway network, the Wnt and SHH pathway components are seen as clear separate modules with a few molecules linking these two modules/pathways. A significant finding in the pathway network (Fig. 2A–C) is that glioma-associated oncogene 2 (Gli2) is one of the molecules connecting the two pathways together, and appears to be the central connector. It is connected to SMO, RAB23 in SHH pathway and to GSK3β, CSNK1A1 and CSNK1G1 in the Wnt pathway (Fig. 2a). While Gli2 is a primary regulator
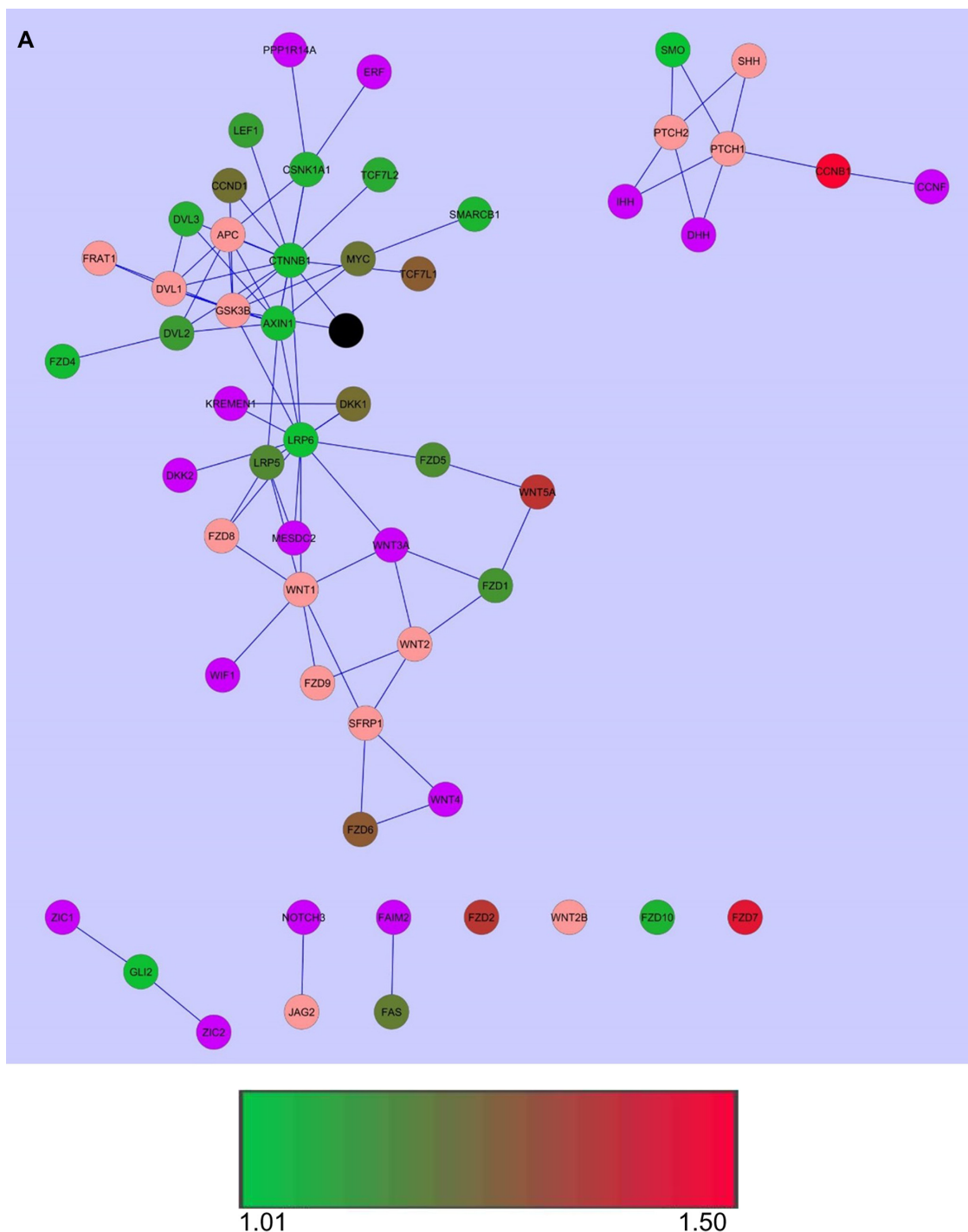
Notes: The color gradient from green to red denotes lower to higher expression values, black node: CDH1, purple-colored nodes: proteins encoded by genes not present in the gene list under study, but automatically added as neighboring proteins in PPI network, pink-colored nodes: under-expressed or not expressed in a particular type of sample.
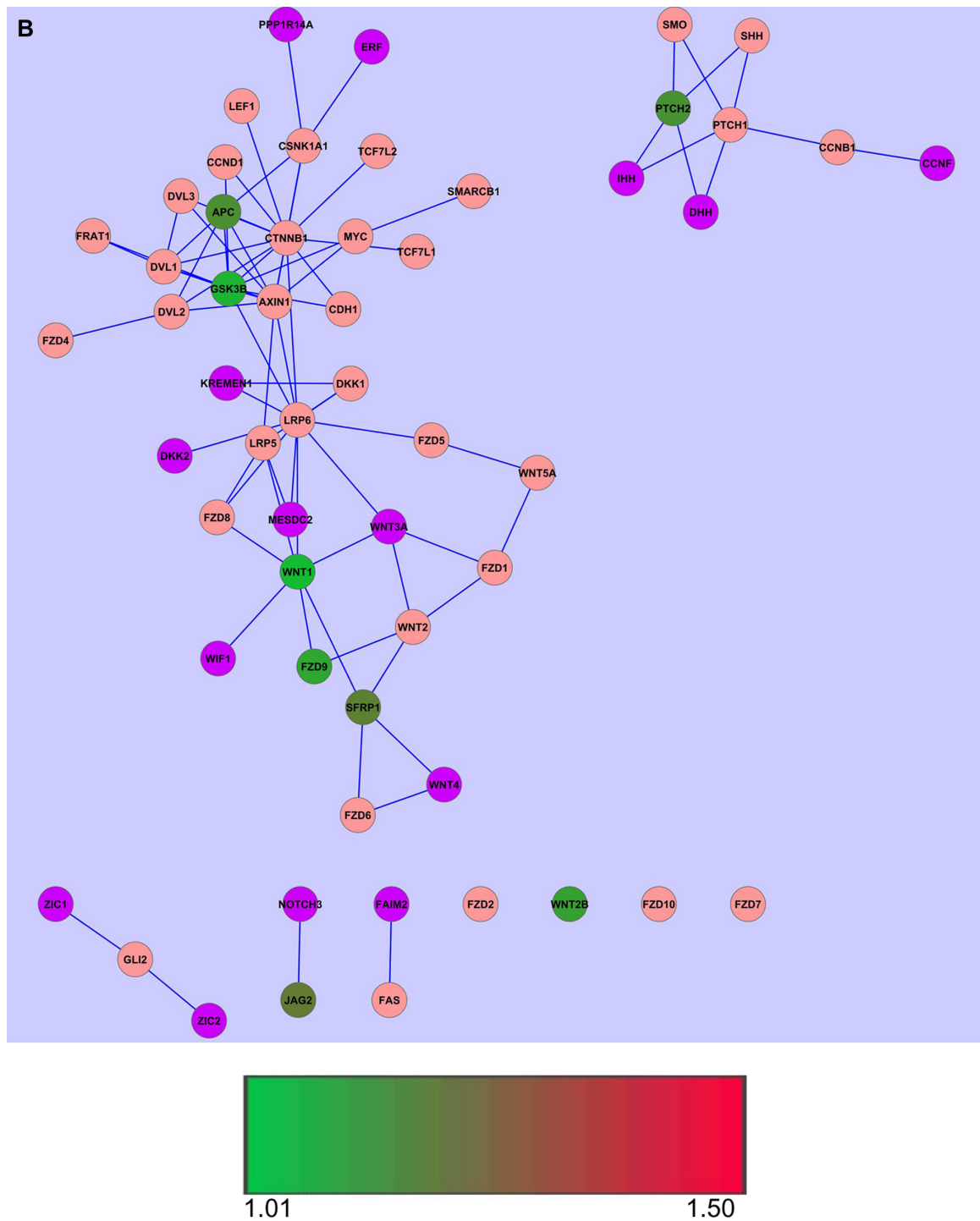
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in SHH signaling and mainly functions as a transcriptional activator, it also regulates Wnt genes involved in morphogenesis. Even though it is not yet found to be involved in PPI with other molecules in the gene list in the present paper as seen in PPI network (Fig. 1), due to its significant differential expression in GBM coupled with the fact that it acts as a bridge connecting SHH and Wnt signaling in the pathways network, it is highly probable that it plays a central role. In fact, Gli2, by dint of being a connector molecule may play a much bigger role than is generally anticipated and studies should be directed toward expanding its functional role. PubMed search as on January 30, 2014 indicates that there are a total of just 613 studies covering Gli2 molecule, even as its name implies its possible crucial role in glioma development.

Figure 1. (*Continued*)

Notes: The color gradient from green to red denotes lower to higher expression values, black node: CDH1, purple-colored nodes: proteins encoded by genes not present in the gene list under study, but automatically added as neighboring proteins in PPI network, pink-colored nodes: under-expressed or not expressed in a particular type of sample.

As such, this study points out that Gli2 upregulation may be correlated with GBM progression. Since Gli2 degradation occurs *via* GSK3β-dependent phosphorylation and ubiquitination, increasing the activity of GSK3β may be one potential mechanism of therapy. What is more conclusive is that, GSK3β is found upregulated in normal tissues and not in tumors, hence Gli2 is not degraded in tumors, and so, may play a pro-active role in GBM tumor development.
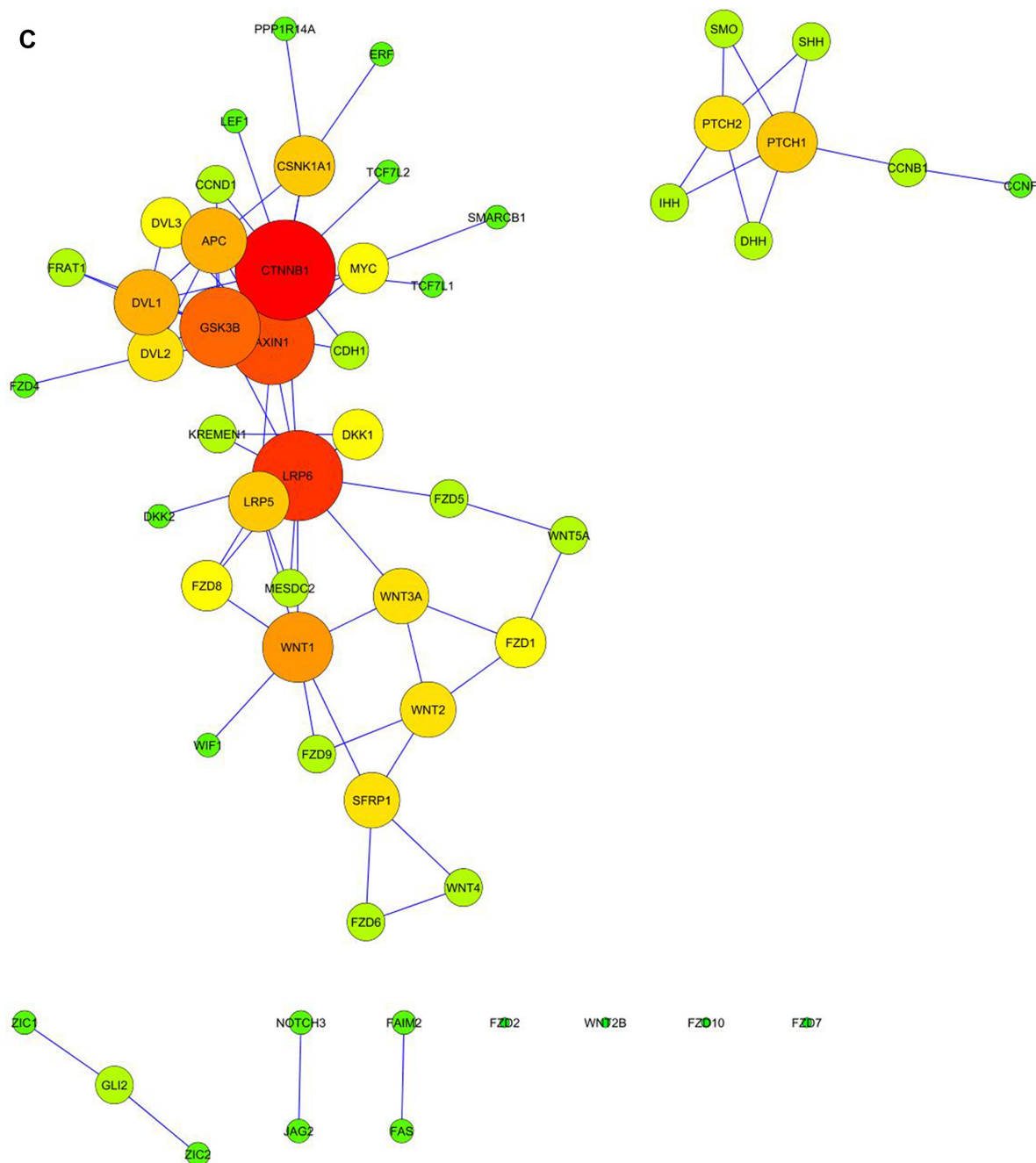
Figure 1. (*Continued*)

Figure 1. PPI networks overlaid with gene expression data. (**A**) PPI networks were overlaid with gene expression data for each gene in tumors. (**B**) PPI networks were overlaid with gene expression data for each gene in normal tissues. Significantly differentially expressed nodes are colored based on expression values. (**C**) Nodes in PPI network sized and colored according to node degree distribution, bigger size of a node corresponds to higher node degree, while the color gradient from green to yellow to red denotes lower to higher node degrees.

Another molecule that appears to connect the two pathways is CSNK1A1 (Fig. 2B), and is in focus because of its significant differential expression and high node degree in PPI network overlaid with gene expression data from tumors (Fig. 1a and c). It is connected to both Gli2 and CTNNB1 in pathway network. CSNK1A1 phosphorylates CTNNB1 in Wnt pathway and SMO in SHH pathway, thereby inactivating these proteins. The mechanism by which CTNNB1 and SMO proteins are prevented from inactivation or remain activated in the presence of high levels of CSNK1A1 in GBM tumors is a matter of further experimental investigation. However, the emerging patterns in this study point to a possible antagonistic role of Gli2 in this mechanism as is explained in "Insights from key emerging patterns" section.

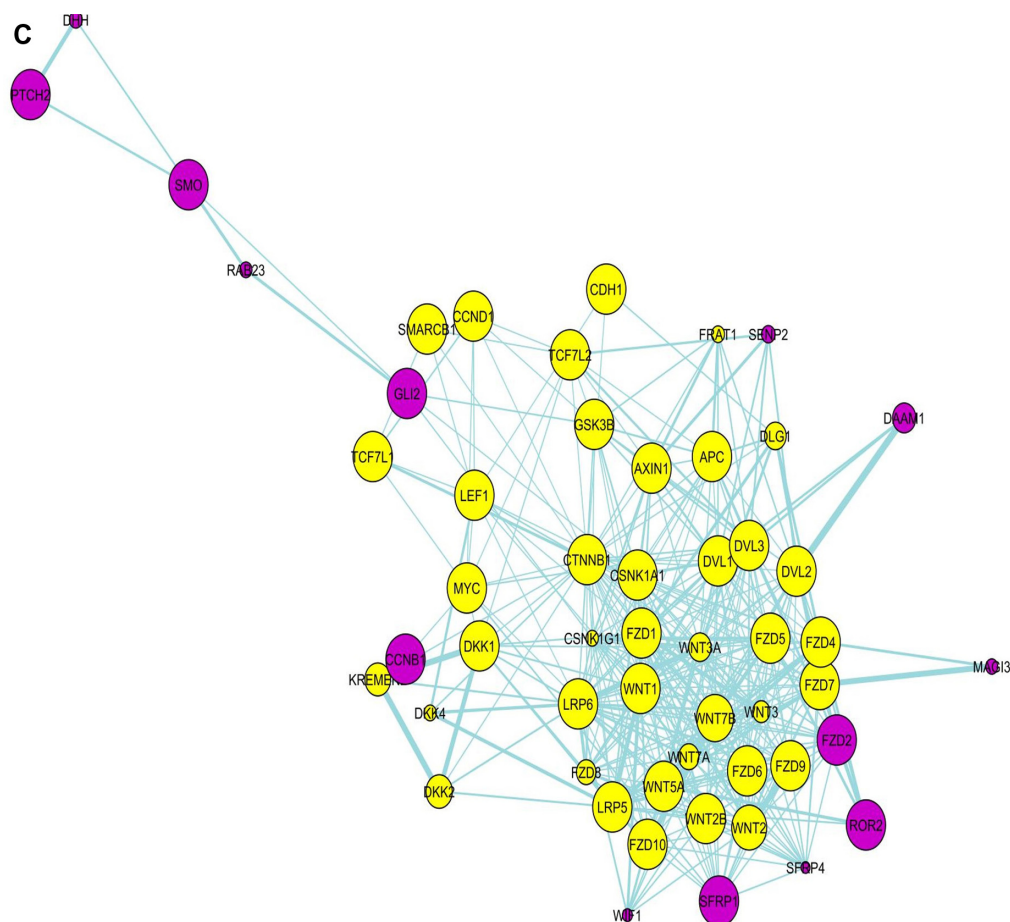The gene or protein expression levels of CTNNB1, CSNK1A1, and Gli2 have been reported as prognostic and

A

B

(*Continued*)

Figure 2. (*Continued*)

Figure 2. Pathway network involving the Wnt- and SHH pathway molecules. Gli2 appears as the connector molecule of Wnt- and SHH pathway in this network, connected to CSNK1A1 and others in Wnt pathway network, and SMO and others in SHH pathway network. Yellow-colored nodes are the first neighbors (directly connected) of (a) Gli2, (b) CSNK1A1, and (c) CTNNB1.

predictor factors in several types of tumors. CTNNB1 and Gli1 are found to serve as prognostic markers in GBM.[23] Significant correlation was observed between high β-catenin (CTNNB1) activity and poor prognosis of the patients, and this was considered as "a strong and independent prognostic factor in breast cancer."[24] CTNNB1 has also been found to serve as a useful prognostic marker in non-small cell lung cancer and gastric cancer[25,26] and in pair with CSNK1E, a prognostic marker in colorectal cancer.[27]

CSNK1A1 has been reported to be overexpressed at both mRNA and protein levels in melanoma cells as compared to normal cells leading to the proposition that it can serve as a useful diagnostic marker.[28] High Gli2 protein expression level in hepatocellular carcinoma (HCC) was found to be associated with poor prognosis in HCC patients after hepatectomy[29] and in the case of intrahepatic cholangiocellular carcinoma (ICC) was found to be associated with unfavorable overall survival prognosis.[30] The gene expression (mRNA expression) level of Gli2 was found to be a negative prognostic factor in acute myeloid leukemia (AML).[31]

Observed among the immediate neighbors of Wnt5a in the pathway network are Fzd4 and LRP5. In the presence of these components, Wnt5a is able to activate the canonical Wnt/β-catenin pathway[19] and may be functioning in the same manner in GBM.

Bottleneck nodes. Betweenness centrality measure is a more significant indicator that a gene/gene product is essential to the proper functioning of a pathway network. This is measured in terms of those network nodes that have many shortest paths going through them, and the nodes with higher betweenness centrality are termed as "bottleneck" nodes.[14] Bottleneck nodes are key connector nodes in a network. As an example, a transcription factor regulating several target genes may function as a bottleneck node in a regulatory network. A key protein/s that can co-ordinate two or more signal transduction pathways is another example of a bottleneck.

Using the convention based on Figure 3 for classification of nodes depending upon "hubness" and "betweenness," it was observed that CTNNB1 and CSNK1A1 fit perfectly as hub–bottleneck nodes, and Gli2 as non-hub–bottleneck node connecting the two major pathways in this study (Fig. 4). The
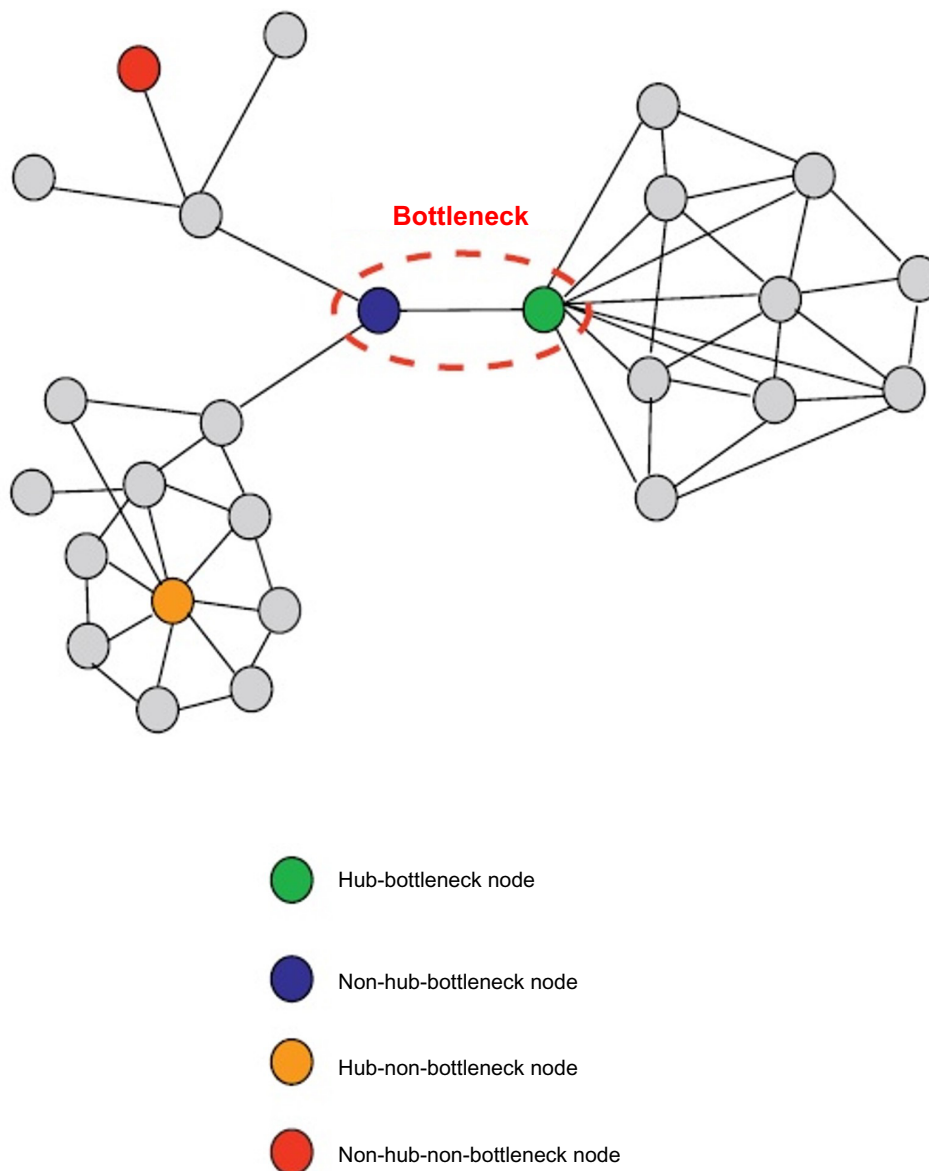
Figure 3. Schematic depiction of bottleneck nodes. Reproduced with permission from Ref. 14.

node with the highest betweenness centrality in Figure 4 is, obviously, CTNNB1. The bottleneck proteins have been found to be essential proteins in both interaction and regulatory networks with high significance.[14] CTNNB1 and CSNK1A1 are well documented to be essential proteins in regulating Wnt and SHH pathways. Non-hub–bottlenecks that are involved in signal transduction pathways are also surmised to be products of essential genes. In this respect, Gli2 as a non-hub bottleneck node may be a gene essential to the overall functioning and cross-talk between these two major pathways. Connecting major pathways together, bottleneck proteins are in a state of dynamic flux for most of the time. Therefore, these are usually significantly co-expressed to a lesser degree with their neighbors and have fewer binding partners than most other nodes in the network, as is observed in the case of Gli2 in co-expression network (data not shown) and Figure 1a, respectively. The "Insights from key emerging

patterns" section details the important roles these three proteins can play as potential therapeutic drug targets.

Insights from key emerging patterns. Combining and integrating all of the above analyses, the picture is becoming clearer. Wnt pathway has emerged as a relatively stronger contender for involvement in the development and progression of GBM as compared to SHH pathway. SHH pathway, through the upregulation and connectivity of some of its gene/gene products to molecules in Wnt pathway, may be playing a helper role in GBM development, at those stages where Wnt pathway might face the roadblocks of inactivation or regulated activation. Even though SHH ligand is not found to be significantly differentially expressed, this pathway is able to survive in GBM. Most likely, this is not due to a ligand-independent aberrant activation, but by some other mechanism involving Wnt pathway molecules in view of SHH pathway playing a helper role, thus creating inter-
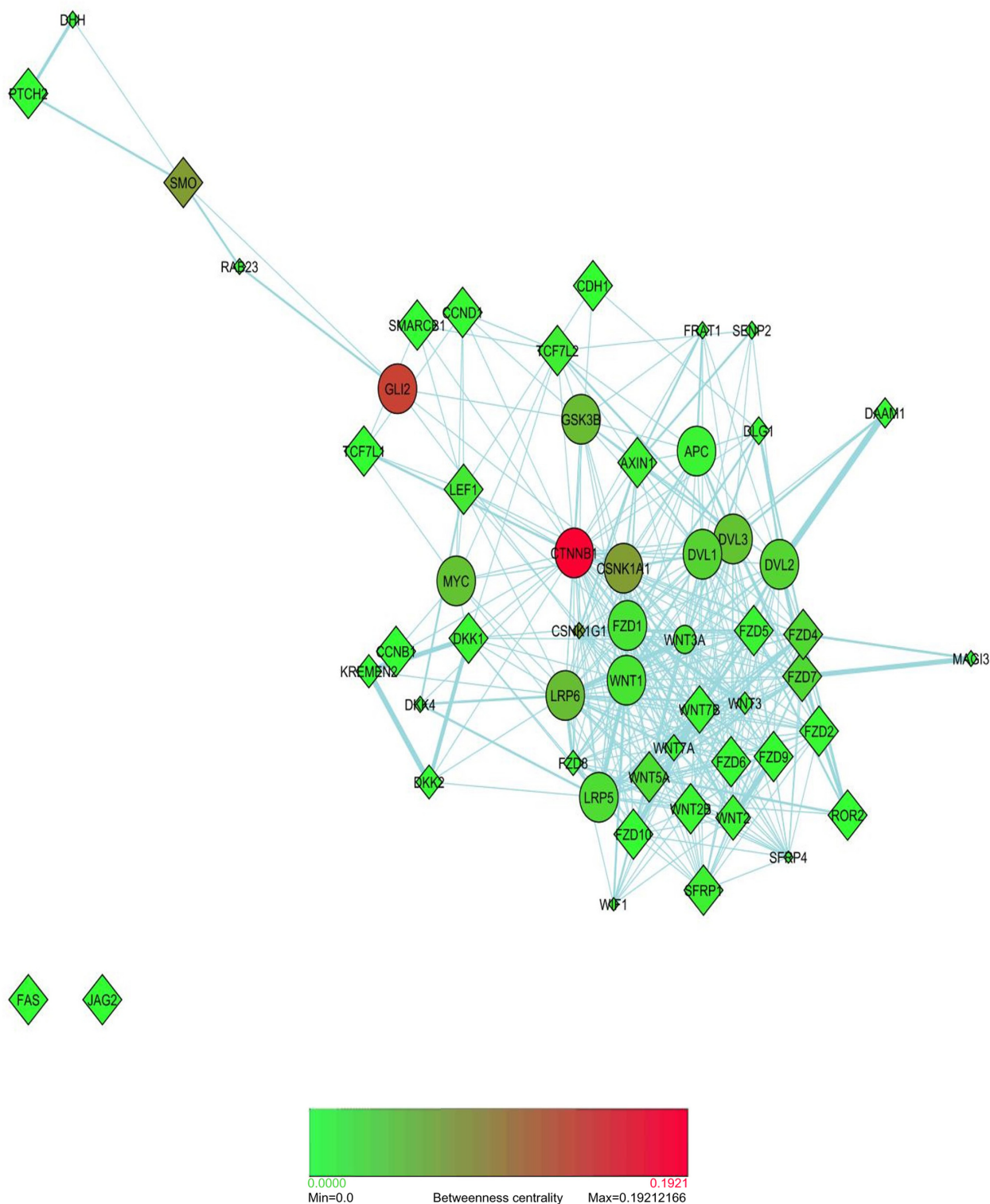
Figure 4. Bottleneck nodes discovered in this study. Nodes in pathway network are colored by betweenness centrality measure.
Notes: The color gradient from green to red denotes lower to higher betweenness centrality, and nodes with higher betweenness centrality are the bottleneck nodes.

dependencies amongst the two. Wnt5a molecule may be the major player in the aberrant activation of both Wnt canonical and non-canonical pathways. Further, in the PPI network, those genes that are not significantly differentially expressed, but are surrounded by genes that are significantly differen-

tially expressed may also be disease associated. An example here is Fzd8, which does not appear to be significantly differentially expressed in this study, but nevertheless, may be playing an active role in GBM development solely due to its connectivity to significantly differentially expressed proteins
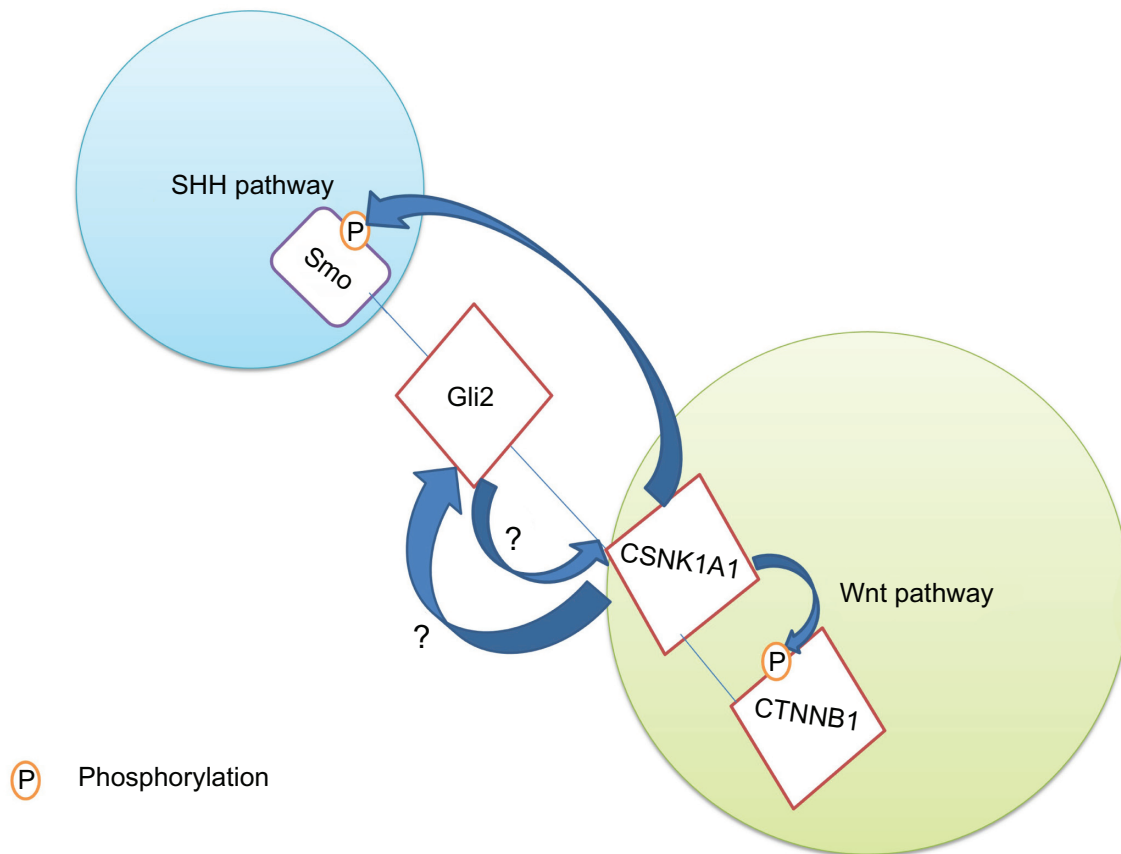
Figure 5. A schematic model of Wnt- and SHH pathways working interdependently in GBM based upon observations in this study. As observed from PPI network and betweenness centrality measures, CSNK1A1 molecule is directly connected to both Gli2 in SHH pathway and CTNNB1 in Wnt pathway, all these three molecules having high betweenness centrality. These are considered as plausible drug targets based on this study and denoted as diamond-shaped nodes. CSNK1A1 is indirectly connected to SMO in SHH pathway. The arrows indicate that the overexpression of CSNK1A1 leads to phosphorylation of CTNNB1 and SMO (indicated by "P" in the nodes), thereby inactivating these two pathways, for which evidence is present in literature. However, the cross-talk between CSNK1A1 and Gli2 is not available to the best of knowledge, and therefore, needs to be studied further. It is surmised that since Wnt and SHH pathways appear to be aberrantly activated in GBMs in this study, despite upregulation and significant differential gene expression of CSNK1A1 in tumors, Gli2 molecule may simply be acting as an antagonist of CSNK1A1. It may diminish the effect of CSNK1A1 on CTNNB1 and SMO, or inhibit CSNK1A1 altogether, leading to aberrant activation of these pathways.

such as LRP5, LRP6, and Wnt1. Bottleneck proteins in a network that connect different functional clusters are more likely to be product of essential genes,[14] which when targeted can lead to the inactivation of all the linked clusters simultaneously. These proteins need not have a high node degree, ie, linked individually to most of the other nodes. In this respect, CSNK1A1, Gli2, and CTNNB1 are prominent in the role of a bottleneck, and therefore, may function as strong drug targets. CSNK1A1, by virtue of it being connected to both Gli2 and CTNNB1, may be a stronger target. In order to serve as a target, it would need to be overexpressed, leading to phosphorylation of CTNNB1 and SMO and subsequent inactivation of the two pathways; this activation, instead of inhibition, of a kinase molecule may present a novel approach in GBM therapy. Indeed, a FDA-approved small-molecule activator of casein kinase 1 alpha, pyrvinium, when used to treat colon cancer cells with mutation in APC or CTNNB1 gene, inhibited both Wnt signaling and proliferation.[32]

To the best of knowledge till date, the interplay between CSNK1A1 and Gli2 molecule is not explored and so, the effect of CSNK1A1 overexpression on Gli2 molecule is open to experimental investigation. While it is entirely possible that Gli2 molecule may also be phosphorylated, leading to its inactivation, it is more likely that Gli2 molecule may act as an antagonist of CSNK1A1. In its antagonistic role, it may diminish the effect of CSNK1A1 on CTNNB1 and SMO, and thereby aberrant activation of these pathways. This may be the reason that despite CSNK1A1 being significantly differentially expressed and upregulated in tumors, Wnt and SHH pathways still proceed as seen from the greater expression of majority of genes in tumors.

GBMs are developing resistance to temozolomide (TMZ) chemotherapy, the main treatment regimen in combination with surgery and radiotherapy. This occurs, in part, due to self-renewal capacity of glioma stem cells. Hh/Gli1 signaling axis controls the behavior of glioma stem cells,[33] and inhibition of SHH path-

way with cyclopamine has been shown to increase the efficacy of TMZ in CD133(+) glioma stem cells.[34] Using Gli2 inhibitor Gant61, or a CTNNB1 inhibitor such as PNU74654 or BC21, or CSNK1A1 activator, pyrvinium, the same approach can be applied to increase the efficacy of TMZ in GBM therapy.

Keeping into account all of these analyses, a schematic model is proposed for the interdependent nature of the two pathways providing us with a new biological insight open to experimentation, as well as a way for simultaneous targeting in GBM (Fig. 5).

Conclusions

Using the mRNA expression patterns of Wnt and SHH pathway genes from TCGA dataset for GBM tumors integrated with interaction networks, several significantly differentially expressed and highly connected genes in the network were identified. The present studies point to the potential major role of CTNNB1, CSNK1A1, and Gli2 in both Wnt and SHH pathways aberrantly activated in GBM. Further, this integrative analysis suggests these molecules as potential therapeutic drug targets to inhibit/inactivate these pathways simultaneously. While CTNNB1 has been studied extensively as a therapeutic target, CSNK1A1 and Gli2 are found to be relatively novel and to the best of the knowledge of this author, not discovered in the context of GBM before. The interplay between CSNK1A1 and Gli2 needs to be discerned, and hence, more studies should be directed toward this end. It is speculated from the patterns derived from this study that CSNK1A1 may be antagonized by Gli2, leading to aberrant activation of Wnt and SHH signalling pathways. In their respective capacities as potential druggable targets, CTNNB1 and Gli2 need to be inhibited while CSNK1A1 requires itself to be activated. The drug-dependent activation of a kinase molecule is uncommon, and therefore, paves the avenue for novel approaches toward drug design in GBM tumors.

Acknowledgments

Author Contributions

Conceived and designed the experiments: SM. Analyzed the data: SM. Wrote the first draft of the manuscript: SM. Made critical revisions: SM. The author reviewed and approved of the final manuscript.

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