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(54) **Title:** METHODS AND COMPOSITIONS FOR INHIBITING GLIOMA GROWTH

(57) **Abstract:** This invention provides cellular factors that positively regulate glioma growth. The invention provides methods and pharmaceutical compositions for inhibiting glioma growth and treating gliomas in human or non-human subjects. The invention also provides methods of screening for novel compounds that inhibit glioma growth.

WO 2006/128063 A2

METHODS AND COMPOSITIONS FOR INHIBITING GLIOMA GROWTH

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application No. 60/684,352, filed May 25, 2005. The disclosure of the priority application is incorporated herein by reference in its entirety and for all purposes.

FIELD OF THE INVENTION

[0002] The present invention generally relates to inhibition of growth of glioma cells and treatment of glioma. More particularly, the invention pertains to identification of molecules that positively regulate glioma growth and to methods of employing these molecules to screen for anti-glioma agents. The invention also relates to methods of inhibit glioma growth by targeting these molecules.

BACKGROUND OF THE INVENTION

[0003] Gliomas are neuroectodermal tumors of neuroglial origin. They include astrocytoma, oligodendroglioma and ependymoma which are derived from astrocytes, oligodendrocytes and ependymal cells respectively. Gliomas are the most common primary tumors in the brain and are divided into grades I-IV on the basis of their histology and prognosis. About 30,000 patients in US are diagnosed with glioma each year. All gliomas infiltrate the adjacent brain tissue, but they do not metastasise. Abnormalities in receptor tyrosine kinase pathways and loss of tumor suppressor genes are critical in the transformation and growth of malignant gliomas.

[0004] Despite extensive research, the treatment options for gliomas remain very limited (e.g., adjuvant therapy and radiotherapy). There is an unfulfilled need in the

art for effective drugs that treat glioma. The instant invention addresses this and other needs.

SUMMARY OF THE INVENTION

[0005] In one aspect, the invention provides methods for inhibiting or ameliorating growth of glioma tumor cells. The methods entail contacting the glioma tumor cells with an agent which down-regulates expression or cellular level of a positive growth regulator of glioma encoded by a polynucleotide selected from the members listed in Table 1. In some of the methods, the agent down-regulates a positive growth regulator of glioma selected from the group consisting of CDC25B, NEK9, KIAA0703, ABL1, ING4 and CBLC. Some of the methods are directed to inhibit growth of glioma tumor cells that are present in a subject. In some methods, the agent is a short interfering RNA (siRNA) that specifically targets the positive growth regulator of glioma.

[0006] In a related aspect, the invention provides methods of inhibiting or ameliorating growth of glioma tumor cells. These methods involve contacting the glioma tumor cells with an agent which down-regulates a biological activity of a positive growth regulator of glioma encoded by a polynucleotide selected from the members listed in Table 1. In some of the methods, the positive growth regulator of glioma is an enzyme, and the agent down-regulates the enzymatic activity of the positive growth regulator of glioma.

[0007] In another aspect, the invention provides methods for identifying agents that inhibit glioma growth. These methods entail first screening test compounds to identify one or more modulating compounds that down-regulate a biological activity or expression of a positive growth regulator of glioma encoded by a polynucleotide selected from the members listed in Table 1, and then testing the modulating compounds for ability to inhibit glioma growth. In some of the methods, the positive growth regulator of glioma employed in the screening is CDC25B, NEK9, KIAA0703, ABL1, ING4 or CBLC.

[0008] A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and claims.

DETAILED DESCRIPTION

I. Overview

[0009] The invention is predicated in part on the discoveries by the present inventors of novel genes that play a positive role in glioma growth. As detailed in the Example below, the present inventors screened a focused siRNA library (Qiagen) in order to identify genes whose knockdown leads to growth inhibition of a glioma cell line. A number of genes were identified from the screening. Knockdown by siRNAs against these genes resulted in at least 2 folds of growth inhibition of glioma cells. To identify genes that are likely to function specifically in glioma cells, several of these hits were further examined for cytotoxicity on normal control cells. siRNAs against a few of the hits exhibited efficacy preferentially in glioma cells that was much greater than their cytotoxic effect in the controls cells.

[0010] The genes identified in the siRNA screening, termed herein "positive growth regulators of glioma," are shown in Table 1. By targeting these genes that positively regulate glioma growth, the invention provides methods and compositions for inhibiting proliferation of glioma cells and for treating glioma in human or non-human subjects. In addition, the genes which play a positive role in glioma growth also provide novel targets to screen for compounds that inhibit glioma growth. The following sections provide further guidance for practicing the therapeutic and screening methods of the invention.

Table 1. Positive growth regulators of glioma identified from siRNA screening

	Acc. No.	Hit Symbol	Description	x fold of modulation
1	NM_001873	CPE	carboxypeptidase E	-2.03737
2	NM_018401	HSA250839	gene for serine/threonine protein kinase	-2.05476
3	NM_016162	ING4	inhibitor of growth family, member 4	*-2.06302*
4	NM_006573	TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	-2.0645
5	XM_113912.1	LOC201140	similar to DKFZP566O084 protein	-2.10509
6	NM_014847	NICE-4	NICE-4 protein	-2.12928
7	NM_021624	HRH4	histamine receptor H4	-2.13152
8	NM_032121	DKFZp564K 142	implantation-associated protein	-2.141
9	XM_293555.2	LOC344715	similar to myosin light chain kinase isoform 3A	-2.14267
10	NM_002022	FMO4	flavin containing monooxygenase 4	-2.17471
11	NM_002988	CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	-2.18088
12	NM_000221	KHK	ketohexokinase (fructokinase)	-2.28069
13	NM_006215	SERPINA4	serine (or cysteine) proteinase inhibitor, clade A	-2.28601

14	NM_022817	PER2	(alpha-1 antiproteinase, antitrypsin), member 4 period homolog 2 (Drosophila)	-2.30634
15	NM_017593	BMP2K	BMP2 inducible kinase	-2.325
16	NM_006668	CYP46A1	cytochrome P450, family 46, subfamily A, polypeptide 1	-2.32979
17	NM_000797	DRD4	dopamine receptor D4	-2.34049
18	NM_002996	CX3CL1	chemokine (C-X3-C motif) ligand 1	-2.36678
19	NM_002014	FKBP4	FK506 binding protein 4, 59kDa	-2.37651
20	NM_001470	GABBR1	gamma-aminobutyric acid (GABA) B receptor, 1	-2.43582
21	NM_014648	DZIP3	zinc finger DAZ interacting protein 3	-2.4887
22	NM_033115	MGC16169	hypothetical protein MGC16169	-2.49285
23	NM_003814	ADAM20	a disintegrin and metalloproteinase domain 20	-2.54386
24	NM_016299	HSP70-4	likely ortholog of mouse heat shock protein, 70 kDa 4	-2.57594
25	NM_013262	MIR	myosin regulatory light chain interacting protein	-2.5913
26	NM_005536	IMPA1	inositol(myo)-1(or 4)-monophosphatase 1	-2.64129
27	NM_014861	KIAA0703	KIAA0703 gene product	-2.6716
28	NM_005157	ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1	-2.69856
29	NM_006082	K-ALPHA-1	tubulin, alpha, ubiquitous	-2.71252
30	NM_015170	SULF1	sulfatase 1	-2.72883
31	NM_032147	USP44	ubiquitin specific protease 44	-2.7406
32	NM_000043	TNFRSF6	tumor necrosis factor receptor superfamily, member 6	-2.77862
33	NM_001648	KLK3	kallikrein 3, (prostate specific antigen)	-2.7976
34	NM_002420	TRPM1	transient receptor potential cation channel, subfamily M, member 1	-2.8441
35	XM_290552.1	CNGA4	cyclic nucleotide gated channel alpha 4	-3.06313
36	NM_007196	KLK8	kallikrein 8 (neuropsin/ovasin)	-3.06859
37	NM_004983	KCNJ9	potassium inwardly-rectifying channel, subfamily J, member 9	-3.17914
38	NM_015690	STK36	serine/threonine kinase 36 (fused homolog, Drosophila)	-3.38923
39	NM_020666	CLK4	CDC-like kinase 4	-3.40308
40	NM_016261	TUBD1	likely ortholog of mouse tubulin, delta 1	-3.45015
41	NM_183378	OVCH1	ovochymase 1	-3.47272
42	NM_005625	SDCBP	syndecan binding protein (syntenin)	-3.55912
43	NM_012116	CBLC	Cas-Br-M (murine) ecotropic retroviral transforming sequence c	-3.57772
44	NM_033116	NEK9	NIMA (never in mitosis gene a)- related kinase 9	-3.6199
45	NM_182488	USP12L1	ubiquitin specific protease 12 like 1	-3.68287
46	NM_018108	C14orf130	chromosome 14 open reading frame 130	-3.7641
47	NM_030755	TXNDC	thioredoxin domain containing	-3.77661
48	NM_000497	CYP11B1	cytochrome P450, family 11, subfamily B, polypeptide 1	-3.88462
49	NM_004358	CDC25B	cell division cycle 25B	-3.95098
50	NM_005811	GDF11	growth differentiation factor 11	-4.0265
51	XM_114089.3	LOC200008	hypothetical protein LOC200008	-4.07751
52	NM_001872	CPB2	carboxypeptidase B2 (plasma, carboxypeptidase U)	-4.15689
53	NM_000746	CHRNA7	cholinergic receptor, nicotinic, alpha polypeptide 7	-4.32739
54	NM_002691	POLD1	polymerase (DNA directed), delta 1, catalytic subunit 125kDa	-4.46533
55	NM_000021	PSEN1	presenilin 1 (Alzheimer disease 3)	-4.49344
56	NM_005700	DPP3	dipeptidylpeptidase 3	-4.56939

57	NM_031464	RPS6KL1	ribosomal protein S6 kinase-like 1	-4.61994
58	NM_024417	FDXR	ferredoxin reductase	-4.68873
59	NM_000807	GABRA2	gamma-aminobutyric acid (GABA) A receptor, alpha 2	-5.421
60	NM_020395	LOC57117	hypothetical nuclear factor SBBI22	-5.58397
61	NM_005574	LMO2	LIM domain only 2 (rhombotin-like 1)	-5.79855
62	NG_002764	IL9RP4	interleukin 9 receptor pseudogene 4	-5.92774
63	NM_017490	MARK2	MAP/microtubule affinity-regulating kinase 2	-5.97971
64	NM_000927	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	-6.53534
65	NM_006217	SERPINI2	serine (or cysteine) proteinase inhibitor, clade I (neuroserpin), member 2	-10.653
66	NM_000775	CYP2J2	cytochrome P450, family 2, subfamily J, polypeptide 2	-20.6105
67	NM_018955	UBB	ubiquitin B	-36.6811

II. Definitions

[0011] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: *Oxford Dictionary of Biochemistry and Molecular Biology*, Smith et al. (eds.), Oxford University Press (revised ed., 2000); *Dictionary of Microbiology and Molecular Biology*, Singleton et al. (Eds.), John Wiley & Sons (3rd ed., 2002); and *A Dictionary of Biology (Oxford Paperback Reference)*, Martin and Hine (Eds.), Oxford University Press (4th ed., 2000). In addition, the following definitions are provided to assist the reader in the practice of the invention.

[0012] The term "agent" or "test agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., protein, polypeptide, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent", "substance", and "compound" can be used interchangeably.

[0013] The term "analog" is used herein to refer to a molecule that structurally resembles a reference molecule but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the reference molecule, an analog would be expected, by one skilled in the art, to exhibit the same, similar, or improved utility. Synthesis and screening

of analogs, to identify variants of known compounds having improved traits (such as higher binding affinity for a target molecule) is an approach that is well known in pharmaceutical chemistry.

[0014] As used herein, "contacting" has its normal meaning and refers to combining two or more molecules (e.g., a compound and a polypeptide) or combining molecules and cells (e.g., a compound and a cell). Contacting can occur in vitro, e.g., combining two or more compounds or combining a test compound and a cell or a cell lysate in a test tube or other container. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate.

[0015] A "heterologous sequence" or a "heterologous polynucleotide," as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous polynucleotide in a host cell includes a polynucleotide that, although being endogenous to the particular host cell, has been modified. Modification of the heterologous sequence can occur, e.g., by treating the polynucleotide with a restriction enzyme to generate a polynucleotide fragment that is capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous polynucleotide.

[0016] The term "homologous" when referring to proteins and/or protein sequences indicates that they are derived, naturally or artificially, from a common ancestral protein or protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity is routinely used to establish homology. Higher levels of sequence similarity, e.g., 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more can also be used to establish homology.

[0017] A "host cell," as used herein, refers to a prokaryotic or eukaryotic cell into which a heterologous polynucleotide (e.g., an expression vector) is to be introduced.

The heterologous polynucleotide can be introduced into the host cell by any means, e.g., transfection, electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and/or the like.

[0018] The term "positive growth regulator of glioma" refers to molecules which are identified by the present inventors to play a positive role in glioma growth and proliferation. The term encompasses genes shown in Table 1 and their encoded polypeptides. siRNA knockdown of expression of these molecules leads to inhibition of glioma cell growth.

[0019] The term "sequence identity" in the context of two nucleic acid sequences or amino acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. A "comparison window" refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are aligned optimally. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482; by the alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443; by the search for similarity method of Pearson and Lipman (1988) Proc. Nat. Acad. Sci U.S.A. 85:2444; by computerized implementations of these algorithms (including, but not limited to CLUSTAL in the PC/Gene program by Intelligentics, Mountain View, CA; and GAP, BESTFIT, BLAST, FASTA, or TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis., U.S.A.). Alignment can also be performed by inspection and manual alignment.

[0020] A "substantially identical" nucleic acid or amino acid sequence refers to a nucleic acid or amino acid sequence which has at least 90% sequence identity to a reference sequence using the programs described above (e.g., BLAST) using standard parameters. The sequence identity is preferably at least 95%, more preferably at least 98%, and most preferably at least 99%. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over

at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

[0021] The term "modulate" with respect to a biological activity of a reference protein or its fragment refers to a change in the expression level or other biological activities (e.g., enzymatic activities) of the protein. For example, modulation may cause an increase or a decrease in expression level of the reference protein, enzymatic modification (e.g., phosphorylation) of the protein, binding characteristics (e.g., binding to a target polynucleotide), or any other biological, functional, or immunological properties of the reference protein. The change in activity can arise from, for example, an increase or decrease in expression of one or more genes that encode the reference protein, the stability of an mRNA that encodes the protein, translation efficiency, or from a change in other biological activities of the reference protein. The change can also be due to the activity of another molecule that modulates the reference protein (e.g., a kinase which phosphorylates the reference protein). Modulation of a reference protein can be up-regulation (i.e., activation or stimulation) or down-regulation (i.e. inhibition or suppression). The mode of action of a modulator of the reference protein can be direct, e.g., through binding to the protein or to genes encoding the protein, or indirect, e.g., through binding to and/or modifying (e.g., enzymatically) another molecule which otherwise modulates the reference protein.

[0022] The term "antagonizing" or "antagonize" is used in its broadest sense. When used in the context of modulating the positive regulators of glioma described herein, antagonism can include any mechanism or treatment which results in inhibition, inactivation, blocking or reduction in a biological activity (e.g., enzymatic activity) of a positive growth regulator of glioma. It also encompasses any mechanism or treatment which results in a down-regulated expression or cellular level of a gene that encodes the positive growth regulator of glioma.

[0023] The term "subject" includes mammals, especially humans. It also encompasses other non-human animals such as cows, horses, sheep, pigs, cats, dogs, mice, rats, rabbits, guinea pigs, monkeys.

[0024] A "variant" of a reference molecule refers to a molecule substantially similar in structure and biological activity to either the entire reference molecule, or to a

fragment thereof. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of amino acid residues is not identical.

III. Inhibiting Glioma Growth with Agents Antagonizing Positive Growth Regulators of Glioma

[0025] By employing agents which down-regulate the positive regulators of glioma described herein, the invention provides methods and compositions to inhibit or ameliorate proliferation of glioma cells. By inhibiting growth of glioma tumor cells, agents that antagonize a positive growth regulator of glioma are also useful in therapeutic or prophylactic treatment of glioma. They can be readily employed to prevent or treat glioma in various subjects, particularly human subjects. Subjects suffering from low-grade gliomas (grades I and II) and high-grade gliomas (grades III and IV) are all suitable for treatment with these agents. Subjects that are suitable for treatment with the methods of the invention are those who are suffering from various types of glioma (e.g., astrocytoma, oligodendroglioma or ependymoma) or those who are at risk or have a predisposition of developing a glioma tumor. Thus, some therapeutic applications of the invention are directed to treat subjects with an existing glioma. In some other applications, the therapeutic methods and compositions of the invention are employed to prevent tumorigenesis in a subject.

[0026] Typically, the methods involve contacting a cell (e.g., a glioma tumor cell) with an agent which antagonizes one positive growth regulator of glioma disclosed herein (e.g., CDC25B, NEK9 or ABL1). In prophylactic or therapeutic treatment of a glioma tumor in a subject, the methods usually entail administering to the subject in need of treatment a pharmaceutical composition that contains an effective amount of an agent that antagonizes the positive growth regulator of glioma. The agent that antagonizes the positive growth regulator of glioma can be used alone or in conjunction with other known anti-cancer agents to provide synergistic effects in the subject. The agent that antagonizes the positive growth regulator of glioma down-regulates cellular level or inhibits a biological activity (e.g., enzymatic activity) of the positive growth regulator of glioma.

These agents include compounds that can be identified in accordance with the screening methods described below, e.g., small molecule compounds or antibodies (e.g., antagonist antibodies). They also include compounds which specifically inhibit expression or down-regulate cellular level of the positive growth regulator of glioma.

[0027] To specifically inhibit expression or down-regulate cellular level of a positive growth regulator of glioma, nucleic acid agents which antagonize a positive growth regulator of glioma (e.g., CDC25B, NEK9 or ABL1) can be employed. Such nucleic acid agents include small interfering RNA (siRNA), anti-sense nucleic acid, microRNA (miRNA), and synthetic hairpin RNA (shRNA), or complementary DNA (cDNA). In some preferred embodiments, the therapeutic methods rely on RNA interference (RNAi) using siRNAs agents that silence or deplete expression of the positive growth regulator of glioma (e.g., CDC25B, NEK9 or ABL1), as exemplified in the Examples below. RNA interference is the process whereby the introduction of double stranded RNA into a cell inhibits the expression of a gene corresponding to its own sequence. RNAi is usually described as a post-transcriptional gene-silencing (PTGS) mechanism in which double stranded RNA triggers degradation of homologous messenger RNA in the cytoplasm. See, e.g., Elbashir et al., Genes Dev. 15, 188-200, 2001; Elbashir et al., Nature 411: 494498, 2001; and Hutvagner et al., Science 293:834-838, 2001. siRNAs bind to a ribonuclease complex called RNA-induced silencing complex (RISC) that guides the small dsRNAs to its homologous mRNA target. Consequently, RISC cuts the mRNA approximately in the middle of the region paired with the antisense siRNA, after which the mRNA is further degraded. Interference with the function and expression of endogenous genes by double-stranded RNA has been shown in various organisms such as C. elegans as described, e.g., in Fire et al., Nature 391:806-811, 1998; drosophilia as described, e.g., in Kennerdell et al., Cell 95:1017-1026, 1998; and mouse embryos as described, e.g., in Wianni et al., Nat. Cell Biol. 2:70-75, 2000.

[0028] As demonstrated in the Examples below, siRNAs targeting the positive regulators of glioma can be prepared with methods well known in the art. Double-stranded RNA can be synthesized by in vitro transcription of single-stranded RNA read from both directions of a template and in vitro annealing of sense and antisense RNA strands. Double stranded RNA can be introduced into a cell of interest (e.g., tumor cell) or a subject in a

number of different ways. These include, e.g., microinjection, bombardment by particles covered by the dsRNA, soaking the cell in a solution of the dsRNA, electroporation of cell membranes in the presence of the dsRNA, liposome-mediated delivery of dsRNA and transfection mediated by chemicals such as calcium phosphate, viral infection, and transformation. In addition, dsRNA can also be supplied to a cell indirectly by introducing one or more vectors that encode both single strands of a dsRNA (or, in the case of a self-complementary RNA, the single self- complementary strand) into the cell. Preferably, the vector contains 5' and 3' regulatory elements that facilitate transcription of the coding sequence. Single stranded RNA is transcribed inside the cell, and, presumably, double stranded RNA forms and attenuates expression of the target gene.

[0029] All of the methods and techniques needed for performing RNAi are well known in the art. For example, WO 99/32619 (Fire et al., published 1 Jul. 1999) described how to supply a cell with dsRNA by introducing a vector from which it can be transcribed. Other teachings of RNAi are provided in, e.g., Reich et al., Mol Vis. 9:210-6 (2003); Gonzalez-Alegre P et al. , Ann Neurol. 53:781-7 (2003); Miller et al., Proc Natl Acad Sci U S A. (2003); Bidere et al., J Biol Chem., published as manuscript M301911200 (Jun. 2, 2003); Van De Wetering et al., EMBO Rep. 4:609-15 (2003); Miller and Grollman, DNA Repair (Amst) 2:759-63 (2003); Kawakami et al., Nat Cell Biol. 5:513-9 (2003); Abdelrahim et al., Mol Pharmacol. 63:1373-81 (2003); Williams et al., J Immunol. 170:5354- 8 (2003); Daude et al., J Cell Sci. 116:2775-9 (2003); Jackson et al., Nat Biotechnol. 21:635-7 (2003); Dillin, Proc Natl Acad Sci U S A. 100:6289-91 (2003); Matta et al. , Cancer Biol Ther. 2:206-10 (2003); Wohlbold et al., Blood. (2003); Julien and Herr, EMBO J. 22:2360-9 (2003); Scherr et al., Cell Cycle. 2:251-7 (2003); Giri et al., J Immunol. 170:5281-94 (2003); Liu and Erikson, Proc Natl Acad Sci U S A. 100:5789-94 (2003); Chi et al., Proc Natl Acad Sci U S A. 100:6343-6 (2003); Hall and Alexander, J Virol. 77:6066-9 (2003).

[0030] Double stranded RNA can be introduced along with components that enhance RNA uptake by the cell, stabilize the annealed strands, or otherwise increase inhibition of the target gene. In the case of a cell culture or tissue explant, the cells are conveniently incubated in a solution containing the dsRNA or lipid-mediated transfection. For a subject (e.g., a non-human animal), the dsRNA can be conveniently introduced by

injection or perfusion into a cavity or interstitial space of an organism, or systemically via oral, topical, parenteral (including subcutaneous, intramuscular and intravenous administration), vaginal, rectal, intranasal, ophthalmic, or intraperitoneal administration. In addition, the dsRNA can be administered via and implantable extended release device. Methods for oral introduction include direct mixing of RNA with food of the subject as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the subject to be affected.

[0031] In addition to siRNAs, other nucleic acid agents targeting the positive regulators of glioma can also be employed in the methods of the present invention, e.g., antisense nucleic acids. Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific target mRNA molecule. In the cell, the single stranded antisense molecule hybridizes to that mRNA, forming a double stranded molecule. The cell does not translate an mRNA in this double- stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Antisense methods have been used to inhibit the expression of many genes in vitro. See, e.g., Marcus-Sekura, Anal.Biochem., 172:289-295 (1988); Hambor et al., Proc. Natl. Acad. Sci. U.S.A. 85:4010-4014 (1988)) and in situ (Arima et al., Antisense Nucl. Acid Drug Dev. 8:319-327 (1998); Hou et al.,Antisense Nucl. Acid Drug Dev. 8:295- 308 (1998).

[0032] In addition to silencing or depleting expression of a positive growth regulator of glioma gene, the therapeutic applications of the invention can also employ agents that antagonizes the positive growth regulator of glioma that inhibit a biological activity of the positive growth regulator of glioma protein. These include compounds that can be identified in accordance with the below described screen methods. Suitable agents that antagonizes the positive growth regulator of glioma also include antagonist antibodies which specifically bind to the positive growth regulator of glioma polypeptide and antagonize its biological activity (e.g., kinase activity for NEK9 or ABL1). Monoclonal antibody-based reagents are among those most highly preferred in this regard. Such antagonist antibodies can be generated using methods well known and routinely practiced in the art, e.g., *Monoclonal Antibodies--Production, Engineering And Clinical Applications*, Ritter et al., Eds., Cambridge University Press, Cambridge, UK, 1995; and Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Press, 3rd ed.,

2000. Radiolabeled monoclonal antibodies for cancer therapy, in particular, are well known and are described in, for instance, *Cancer Therapy With Radiolabelled Antibodies*, D. M. Goldenberg, Ed., CRC Press, Boca Raton, Fla., 1995.

[0033] Compounds which down-regulate expression or a biological activity of the positive growth regulator of glioma can be used in conjunction with other therapies. For example, subjects receiving surgery and radiation therapies can also be administered with a pharmaceutical composition of the present invention. In addition, chemotherapy, hormonal therapy and cryotherapy may also be combined with the therapeutic applications of the present invention to treat subjects suffering from cancers. The agents that antagonize the positive growth regulator of glioma can also be used in a subject to prevent tumor growth or treat cancer together with the administration of other therapeutic compounds for the treatment or prevention of these disorders. When an agent that antagonizes a positive growth regulator of glioma is administered together with another anti-cancer agent, the two can be administered in either order or simultaneously. These therapeutic compounds may be chemotherapeutic agents, ablation or other therapeutic hormones, antineoplastic agents, monoclonal antibodies useful against cancers and angiogenesis inhibitors.

[0034] There are many anti-cancer drugs known in the art, e.g., as described in, e.g., *Cancer Therapeutics: Experimental and Clinical Agents*, Teicher (Ed.), Humana Press (1st ed., 1997); and *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, Hardman et al. (Eds.), McGraw-Hill Professional (10th ed., 2001). Examples of suitable anti-cancer drugs include 5-fluorouracil, vinblastine sulfate, estramustine phosphate, suramin and strontium-89. Examples of suitable chemotherapeutic agents include Asparaginase, Bleomycin Sulfate, Cisplatin, Cytarabine, Fludarabine Phosphate, Mitomycin and Streptozocin. Hormones which may be used in combination with the present invention diethylstilbestrol (DES), leuprolide, flutamide, cyproterone acetate, ketoconazole and amino glutethimide.

IV. Screening for Novel Anti-Glioma Agents – General Scheme

[0035] The positive growth regulators of glioma identified by the present inventors provide novel targets to screen for compounds that inhibit glioma growth and proliferation. Various biochemical and molecular biology techniques or assays well known

in the art can be employed to practice the present invention. Such techniques are described in, e.g., *Handbook of Drug Screening*, Seethala et al. (eds.), Marcel Dekker (1st ed., 2001); *High Throughput Screening: Methods and Protocols (Methods in Molecular Biology, 190)*, Janzen (ed.), Humana Press (1st ed., 2002); *Current Protocols in Immunology*, Coligan et al. (Ed.), John Wiley & Sons Inc (2002); Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (3rd ed., 2001); and Brent et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (ringbou ed., 2003).

[0036] Typically, test agents are first assayed for their ability to modulate a biological activity of a positive growth regulator of glioma encoded by the polynucleotides shown in Table 1 ("the first assay step"). Modulating compounds thus identified are then subject to further screening for ability to inhibit glioma growth, typically in the presence of the positive growth regulator of glioma ("the second testing step"). Depending on the positive growth regulator of glioma employed in the method, modulation of different biological activities of the positive growth regulator of glioma can be assayed in the first step. For example, a test agent can be assayed for binding to the positive growth regulator of glioma. The test agent can be assayed for activity to modulate expression of the positive growth regulator of glioma, e.g., transcription or translation. The test agent can also be assayed for activities in modulating the cellular level or stability of the positive growth regulator of glioma, e.g., post-translational modification or proteolysis.

[0037] If the positive growth regulator of glioma has a known biochemical or enzymatic function (e.g., kinase activity or protease activity), the biological activity monitored in the first screening step can also be the specific biochemical or enzymatic activity of the positive growth regulator of glioma. Examples include kinases (e.g., NEK9, ABL1, HSA250839, KHK, BMP2K, STK36, CLK4, RPS6KL1, and MARK2), proteases (e.g., USP44 and USP12L1), phosphatases (e.g., CDC25B and IMPA1), or other enzymes shown in Table 1 (e.g., CDCL, CPE, FMO4, SULF1, DVCH1, CPB2, FDXR, POLD1, and DPP3). Any of these molecules can be employed in the first screening step. Methods for assaying the enzymatic activities of these molecules are well known and routinely practiced in the art. For any positive growth regulator of glioma, the substrate to be used in the screening can be a molecule known to be enzymatically modified by the enzyme (e.g., a kinase), or a molecule that can be easily identified from candidate substrates for a given

class of enzymes. For example, many kinase substrates are available in the art. See, e.g., www.emdbiosciences.com; and www.proteinkinase.de. In addition, a suitable substrate of a kinase can be screened for in high throughput format. For example, substrates of a kinase can be identified using the Kinase-Glo® luminescent kinase assay (Promega) or other kinase substrate screening kits (e.g., developed by Cell Signaling Technology, Beverly, Massachusetts).

[0038] In the screening, test compounds can be screened for ability to either up-regulate or down-regulate a biological activity of the positive growth regulator of glioma in the first assay step. Preferably, compounds which inhibit the biological activity of the positive growth regulator of glioma are selected for further screening. Once test agents that modulate the positive growth regulator of glioma are identified, they are typically further tested for ability to inhibit glioma growth in the second screening step. This usually involves testing the identified modulating compounds for ability to inhibit growth of glioma cells in an in vitro system or an in vivo animal model. This further testing step is often needed to confirm that their modulatory effect on the positive growth regulator of glioma would indeed lead to inhibition of glioma growth.

[0039] In both the first assaying step and the second testing step, either an intact positive growth regulator of glioma, or a fragment thereof, may be employed. Molecules with sequences which are substantially identical to that of the positive growth regulator of glioma can also be employed. Analogs or functional derivatives of the positive growth regulator of glioma could similarly be used in the screening. The fragments or analogs that can be employed in these assays usually retain one or more of the biological activities of the positive growth regulator of glioma (e.g., kinase activity if the positive growth regulator of glioma employed in the first assaying step is a kinase). Fusion proteins containing such fragments or analogs can also be used for the screening of test agents. Functional derivatives of a positive growth regulator of glioma usually have amino acid deletions and/or insertions and/or substitutions while maintaining one or more of the bioactivities and therefore can also be used in practicing the screening methods of the present invention. A functional derivative can be prepared from a positive growth regulator of glioma by proteolytic cleavage followed by conventional purification procedures known to those skilled in the art. Alternatively, the functional derivative can be produced by recombinant

DNA technology by expressing only fragments of a positive growth regulator of glioma that retain one or more of their bioactivities.

V. Test Compounds

[0040] Test agents or compounds that can be screened with methods of the present invention include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines, oligocarbamates, polypeptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Some test agents are synthetic molecules, and others natural molecules.

[0041] Test agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. Combinatorial libraries can be produced for many types of compound that can be synthesized in a step-by-step fashion. Large combinatorial libraries of compounds can be constructed by the encoded synthetic libraries (ESL) method described in WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503 and WO 95/30642. Peptide libraries can also be generated by phage display methods (see, e.g., Devlin, WO 91/18980). Libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts can be obtained from commercial sources or collected in the field. Known pharmacological agents can be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

[0042] Combinatorial libraries of peptides or other compounds can be fully randomized, with no sequence preferences or constants at any position. Alternatively, the library can be biased, i.e., some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in some cases, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, or to purines.

[0043] The test agents can be naturally occurring proteins or their fragments. Such test agents can be obtained from a natural source, e.g., a cell or tissue lysate. Libraries of polypeptide agents can also be prepared, e.g., from a cDNA library commercially available or generated with routine methods. The test agents can also be peptides, e.g., peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides can be digests of naturally occurring proteins, random peptides, or "biased" random peptides. In some methods, the test agents are polypeptides or proteins. The test agents can also be nucleic acids. Nucleic acid test agents can be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. They can also be inhibitory polynucleotides such as siRNA, shRNA, or anti-sense DNA. For example, digests of prokaryotic or eukaryotic genomes can be similarly used as described above for proteins.

[0044] In some preferred methods, the test agents are small molecule organic compounds, e.g., chemical compounds with a molecular weight of not more than about 1,000 or not more than about 500. Preferably, high throughput assays are adapted and used to screen for such small molecules. In some methods, combinatorial libraries of small molecule test agents as described above can be readily employed to screen for small molecule compound that inhibit glioma growth. A number of assays are available for such screening, e.g., as described in Schultz (1998) Bioorg Med Chem Lett 8:2409-2414; Weller (1997) Mol Divers. 3:61-70; Fernandes (1998) Curr Opin Chem Biol 2:597-603; and Sittampalam (1997) Curr Opin Chem Biol 1:384-91.

[0045] Libraries of test agents to be screened with the claimed methods can also be generated based on structural studies of the positive growth regulator of glioma discussed above or their fragments. Such structural studies allow the identification of test agents that are more likely to bind to the positive growth regulator of glioma. The three-dimensional structures of the positive growth regulator of glioma can be studied in a number of ways, e.g., crystal structure and molecular modeling. Methods of studying protein structures using x-ray crystallography are well known in the literature. See Physical Bio-chemistry, Van Holde, K. E. (Prentice-Hall, New Jersey 1971), pp. 221-239, and Physical Chemistry with Applications to the Life Sciences, D. Eisenberg & D. C. Crothers (Benjamin Cummings, Menlo Park 1979). Computer modeling of the structures of positive

growth regulators of glioma provides another means for designing test agents to screen for modulators of glioma growth. Methods of molecular modeling have been described in the literature, e.g., U.S. Patent No. 5,612,894 entitled "System and method for molecular modeling utilizing a sensitivity factor," and U.S. Patent No. 5,583,973 entitled "Molecular modeling method and system." In addition, protein structures can also be determined by neutron diffraction and nuclear magnetic resonance (NMR). See, e.g., Physical Chemistry, 4th Ed. Moore, W. J. (Prentice-Hall, New Jersey 1972), and NMR of Proteins and Nucleic Acids, K. Wuthrich (Wiley-Interscience, New York 1986).

[0046] Modulators of the present invention also include antibodies that specifically bind to a positive growth regulator of glioma listed in Table 1. Such antibodies can be monoclonal or polyclonal. Such antibodies can be generated using methods well known in the art. For example, the production of non-human monoclonal antibodies, e.g., murine or rat, can be accomplished by, for example, immunizing the animal with a positive growth regulator of glioma in Table 1 or its fragment (See Harlow & Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York). Such an immunogen can be obtained from a natural source, by peptides synthesis or by recombinant expression.

VI. Screening for Modulators of Positive growth regulators of glioma

[0047] As noted above, test agents are typically first screened for ability to modulate a biological activity of a positive growth regulator of glioma identified by the present inventors. A number of assay systems can be employed in this screening step. The screening can utilize an in vitro assay system or a cell-based assay system. In this screening step, test agents can be screened for binding to a positive growth regulator of glioma, altering expression level of the positive growth regulator of glioma, or modulating other biological activities (e.g., enzymatic activities) of the positive growth regulator of glioma.

1. modulating binding activities of positive growth regulator of glioma

[0048] In some methods, binding of a test agent to a positive growth regulator of glioma is determined in the first screening step. Binding of test agents to a positive growth

regulator of glioma can be assayed by a number of methods including e.g., labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.), and the like. See, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168; and also Bevan et al., Trends in Biotechnology 13:115-122, 1995; Ecker et al., Bio/Technology 13:351-360, 1995; and Hodgson, Bio/Technology 10:973-980, 1992. The test agent can be identified by detecting a direct binding to the positive growth regulator of glioma, e.g., co-immunoprecipitation with the positive growth regulator of glioma by an antibody directed to the positive growth regulator of glioma. The test agent can also be identified by detecting a signal that indicates that the agent binds to the positive growth regulator of glioma, e.g., fluorescence quenching or FRET.

[0049] Competition assays provide a suitable format for identifying test agents that specifically bind to a positive growth regulator of glioma. In such formats, test agents are screened in competition with a compound already known to bind to the positive growth regulator of glioma. The known binding compound can be a synthetic compound. It can also be an antibody, which specifically recognizes the positive growth regulator of glioma, e.g., a monoclonal antibody directed against the positive growth regulator of glioma. If the test agent inhibits binding of the compound known to bind the positive growth regulator of glioma, then the test agent also binds the positive growth regulator of glioma.

[0050] Numerous types of competitive binding assays are known, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see Stahli et al., Methods in Enzymology 9:242-253, 1983); solid phase direct biotin-avidin EIA (see Kirkland et al., J. Immunol. 137:3614-3619, 1986); solid phase direct labeled assay, solid phase direct labeled sandwich assay (see, Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Press, 3rd ed., 2000); solid phase direct label RIA using ^{125}I label (see Morel et al., Mol. Immunol. 25(1):7-15, 1988); solid phase direct biotin-avidin EIA (Cheung et al., Virology 176:546-552, 1990); and direct labeled RIA (Moldenhauer et al., Scand. J. Immunol. 32:77-82, 1990). Typically, such an assay involves the use of purified polypeptide bound to a solid surface or cells bearing either of these, an unlabelled test agent and a labeled reference compound. Competitive inhibition is measured by determining the

amount of label bound to the solid surface or cells in the presence of the test agent. Usually the test agent is present in excess. Modulating agents identified by competition assay include agents binding to the same epitope as the reference compound and agents binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference compound for steric hindrance to occur. Usually, when a competing agent is present in excess, it will inhibit specific binding of a reference compound to a common target polypeptide by at least 50 or 75%.

[0051] The screening assays can be either in insoluble or soluble formats. One example of the insoluble assays is to immobilize a positive growth regulator of glioma or its fragment onto a solid phase matrix. The solid phase matrix is then put in contact with test agents, for an interval sufficient to allow the test agents to bind. After washing away any unbound material from the solid phase matrix, the presence of the agent bound to the solid phase allows identification of the agent. The methods can further include the step of eluting the bound agent from the solid phase matrix, thereby isolating the agent. Alternatively, other than immobilizing the positive growth regulator of glioma, the test agents are bound to the solid matrix and the positive growth regulator of glioma is then added.

[0052] Soluble assays include some of the combinatory libraries screening methods described above. Under the soluble assay formats, neither the test agents nor the positive growth regulator of glioma are bound to a solid support. Binding of a positive growth regulator of glioma or fragment thereof to a test agent can be determined by, e.g., changes in fluorescence of either the positive growth regulator of glioma or the test agents, or both. Fluorescence may be intrinsic or conferred by labeling either component with a fluorophor.

[0053] In some binding assays, either the positive growth regulator of glioma, the test agent, or a third molecule (e.g., an antibody against the positive growth regulator of glioma) can be provided as labeled entities, i.e., covalently attached or linked to a detectable label or group, or cross-linkable group, to facilitate identification, detection and quantification of the polypeptide in a given situation. These detectable groups can comprise a detectable polypeptide group, e.g., an assayable enzyme or antibody epitope. Alternatively, the detectable group can be selected from a variety of other detectable groups

or labels, such as radiolabels (e.g., ^{125}I, ^{32}P, or ^{35}S) or a chemiluminescent or fluorescent group. Similarly, the detectable group can be a substrate, cofactor, inhibitor or affinity ligand.

2. modulating other activities of the positive growth regulators of glioma

[0054] Binding of a test agent to a positive growth regulator of glioma provides an indication that the agent can be a modulator of the positive growth regulator of glioma. It also suggests that the agent may inhibit glioma growth by acting on the positive growth regulator of glioma. Thus, a test agent that binds to a positive growth regulator of glioma can be tested for effect on glioma growth (i.e., in the second testing step outlined above). Alternatively, a test agent that binds to a positive growth regulator of glioma can be further examined to determine whether it indeed modulates a biological activity (e.g., an enzymatic activity) of the positive growth regulator of glioma. The existence, nature, and extent of such modulation can be tested with an activity assay. More often, such activity assays can be used independently to identify test agents that modulate activities of a positive growth regulator of glioma (i.e., without first assaying their ability to bind to the positive growth regulator of glioma).

[0055] In general, the methods involve adding a test agent to a sample containing a positive growth regulator of glioma in the presence or absence of other molecules or reagents which are necessary to test a biological activity of the positive growth regulator of glioma (e.g., enzymatic activity if the positive growth regulator of glioma is an enzyme), and determining an alteration in the biological activity of the positive growth regulator of glioma. Methods for monitoring various enzymatic activities are well known in the art. For example, in addition to the assays noted above, many other assays for monitoring protein kinase activities are also described in the art. These include assays reported in, e.g., Chedid et al., J. Immunol. 147: 867-73, 1991; Kontny et al., Eur J Pharmacol. 227: 333-8, 1992; Wang et al., Oncogene 13: 2639-47, 1996; Murakami et al., Oncogene 14: 2435-44, 1997; Pyrzynska et al., J. Neurochem.74: 42-51, 2000; Berry et al., Biochem Pharmacol. 62: 581-91, 2001; Cai et al., Chin Med J (Engl). 114: 248-52, 2001. Any of these methods may be employed and modified to assay modulatory effect of a test

agent on a positive growth regulator of glioma that is a kinase, e.g., NEK9, ABL1, HSA250839, KHK, BMP2K, STK36, CLK4, RPS6KL1, and MARK2.

[0056] In some preferred embodiments, the positive growth regulators of glioma used in the screening methods are encoded by genes whose knockdown does not present significant cytotoxicity to non-tumor cells relative to their knockdown on glioma cells. Examples of such positive growth regulators of glioma include NEK9 (Acc. No. NM_033116), CDC25B (Acc. No. NM_004358), CBLC (Acc. No. NM_012116), ABL1 (Acc. No. NM_005157), ING4 (Acc. No. NM_016162) and KIAA0703 (Acc. No. NM_014861). Test compounds can be screened for ability to modulate various biological activities of these molecules or expression of the genes encoding these molecules. For example, if NEK9 is employed in the screening, test compounds can be screening for ability to modulate the kinase activity of NEK9 (e.g., its autophosphorylation). Methods for assaying kinase activity of NEK9 are known in the art, e.g., as described in Tan et al., J. Biol. Chem. 279:9321-9330, 2004; and Roig et al., Genes Dev. 16:1640-58, 2002. Similarly, test compounds can be screened for ability to modulate a biological activity of CDC25B, CBLC, ABL1, ING4 or KIAA0703. For example, they can be examined for activity in monitoring the ubiquitin protein ligase activity of CBLC (see, e.g., Kassenbrock et al., J Biol Chem. 279:28017-27, 2004). Compounds modulating CDC25B can be identified by screening test compounds for ability to modulate the phosphatase activity of CDC25B (see, e.g., Honda et al., FEBS Lett. 318:331-334, 1993; and De Souza et al., Exp Cell Res.;257:11-21, 2000) or phosphorylation of CDC25B by Aurora-A protein kinase (see, e.g., Dutertre et al., J Cell Sci. 117:2523-31, 2004). If ABL1 is employed in the screening, test compounds can be screened for ability to modulate phosphorylation of ABL1 by the CDC2 kinase (see, e.g., Kipreos et al., Science 248:217-20, 1990) or the tyrosine kinase activity of ABL1 (see, e.g., Zhu et al., Mol Cell Biol. 16:7054–7062, 1996; and Wen et al., Genes Dev. 11:2456-67, 1997). Modulating compounds for ING4 can be identified by screening test compounds for activity in modulating ING4 physical interaction with p53 or p300 using methods described in the art (e.g., Shiseki et al., Cancer Research 63, 2373-2378, 2003). Compounds that modulate KIAA0703 can be screened for by monitoring the Ca^{2+}/Mn^{2+}-ATPase activity of KIAA0703 (see, e.g., Xiang et al., J. Biol. Chem. 280:11608-11614, 2005).

[0057] In addition to assays for screening agents that modulate enzymatic or other biological activities of a positive growth regulator of glioma, the activity assays also encompass in vitro screening and in vivo screening for alterations in expression level of the positive growth regulator of glioma. Modulation of expression of a positive growth regulator of glioma can be examined in a cell-based system by transient or stable transfection of an expression vector into cultured cell lines. For example, test compounds can be assayed for ability to inhibit expression of a reporter gene (e.g., luciferase gene) under the control of a transcription regulatory element (e.g., promoter sequence) of a positive growth regulator of glioma. Genes encoding the positive growth regulators of glioma (e.g., CDC25B, NEK9, KIAA0703, ABL1, ING4 and CBLC) have been characterized in the art. Their transcription regulatory elements such as promoter sequences have all been delineated.

[0058] Assay vector bearing the transcription regulatory element that is operably linked to the reporter gene can be transfected into any mammalian cell line for assays of promoter activity. Reporter genes typically encode polypeptides with an easily assayed enzymatic activity that is naturally absent from the host cell. Typical reporter polypeptides for eukaryotic promoters include, e.g., chloramphenicol acetyltransferase (CAT), firefly or Renilla luciferase, beta-galactosidase, beta-glucuronidase, alkaline phosphatase, and green fluorescent protein (GFP). Vectors expressing a reporter gene under the control of a transcription regulatory element of a positive growth regulator of glioma can be prepared using only routinely practiced techniques and methods of molecular biology (see, e.g., e.g., Samrbook et al., *supra*; Brent et al., *supra*). In addition to a reporter gene, the vector can also comprise elements necessary for propagation or maintenance in the host cell, and elements such as polyadenylation sequences and transcriptional terminators. Exemplary assay vectors include pGL3 series of vectors (Promega, Madison, WI; U.S. Patent No. 5,670,356), which include a polylinker sequence 5' of a luciferase gene. General methods of cell culture, transfection, and reporter gene assay have been described in the art, e.g., Samrbook et al., *supra*; and Transfection Guide, Promega Corporation, Madison, WI (1998). Any readily transfectable mammalian cell line may be used to assay expression of the reporter gene from the vector, e.g., HCT116, HEK 293, MCF-7, and HepG2 cells.

VII. Testing Modulating Compounds for Inhibitory Activity on Glioma Growth

[0059] To identify novel anti-glioma agents, compounds that modulate a positive growth regulator of glioma as described above are usually further tested to confirm their inhibitory effect on glioma growth. Typically, the compounds are screened for ability to inhibit an activity that is indicative of glioma growth (e.g., proliferation of a glioma cell). By comparing the activity in the presence or absence of a modulating compound, inhibitory activities of the compounds on glioma growth can be identified. The screening is performed in the presence of the positive growth regulator of glioma on which the modulating compounds act. Preferably, this screening step is performed in vivo using cells that endogenously express the positive growth regulator of glioma. As a control, effect of the modulating compounds on a cell that does not express the positive growth regulator of glioma may also be examined.

[0060] Many assays and methods are available to examine glioma-inhibiting activity of the modulating compounds. Examples include microtitration (MTT) assay (see, e.g., Morgan et al., Br J Cancer. 47:205-14, 1983), colony formation assay (see, e.g., Strauss et al., Cancer Cell Int. 2:2, 2002), a chemosensitivity assay based on the protein staining using sulforhodamine B (see, e.g., Haselsberger et al., Anticancer Drugs 7:331-8, 1996), and trypan blue dye exclusion assay (see, e.g., Iida et al., J Cancer Res Clin Oncol. 123:619-22, 1997). These assays usually involve testing the compounds for ability to inhibit growth of cultured glioma cells in vitro. Typically, the cells are first contacted with a modulating compound. Following an incubation period, effect of the compound on glioma growth is examined by measuring the number of viable cells in the culture. As a control, cells not contacted with the compound or contacted with a control compound is also grown under the same growth condition followed by quantification of cell growth.

[0061] Many glioma cells are available and can be employed in the screening. These include glioma cell lines derived human gliomas as well as glioma cell lines established from other species. For example, human glioma cell lines such as A172, U373, U138, U87, and SW1783 (Balzarotti et al., Oncol Res. 14:325-30, 2004) can be readily employed in the screening methods of the invention. Many other suitable human glioma cell lines that can be used are also described in the art, e.g., Zhang et al., Neuropathol. 25:136-43, 2005; and Iida et al., J Cancer Res Clin Oncol. 123:619-22, 1997. Examples of

suitable cell lines from non-human species include mouse glioma cell line, e.g., the Gl261 cell line (Shapiro et al., Cancer Res 30: 2401-2413, 1970), or rat glioma cell lines, e.g., CNS1 and C6 cell lines (Boussif et al., Proc Natl Acad Sci USA 1995; 92: 7297-7301, 1995).

[0062] In some preferred methods, effect of the modulating compounds on glioma growth is assessed by examining viability of cultured human glioma cells in parallel format. For example, human glioma cells, e.g., A172 cell line, can be grown in the wells of culture plates in the presence of one different modulating compound in each well. Following incubation with the compounds, viability of the cells can be examined by a CellTiter-Glo luminescent cell viability assay (Riss et al., Promega Notes 81:2–5, 2002). As demonstrated in the Example below, this assay is a very sensitive, routinely practiced method for assaying cell proliferation and cytotoxicity. It uses a unique, stable form of luciferase to measure ATP as an indicator of viable cells. The luminescent signal produced is proportional to the number of viable cells present in culture. It is also well suited for high-throughput applications and is scalable from 96-, 384- and 1536-well formats. Relative to controls (e.g., cells not contacted with any compound), a reduction in the number of viable cells following due to the presence of a modulating compound indicates an inhibitory activity of the compound on glioma cell growth.

[0063] In some methods, potential inhibitory activity of the modulating compounds on glioma growth can be examined with a colony formation assay as described in the art, e.g., Hong et al., Cancer Res. 65:3617-23, 2005; Alaminos et al., Cancer Res. 65:2565-71, 2005; Hiratsuka et al., Biochem Biophys Res Commun. 309:558-66, 2003; and Strauss et al., Cancer Cell Int. 2:2, 2002. For example, glioma cells can be grown in the wells of culture plates. After treating glioma cells with the compounds, cells can then be fixed (e.g., in methanol) and stained (e.g., with crystal violet). Cytotoxic effect of the compounds on the glioma cells can be determined by quantifying the numbers of viable cells in the colonies.

[0064] In some other methods, inhibitory activity of the modulating compounds on glioma cell proliferation is assessed with a radioactive or colorimetric microtitration (MTT) assay (see, e.g., Morgan et al., Br J Cancer. 47:205-14, 1983; and Fehlauer et al., J Cancer Res Clin Oncol. 126:711-6, 2000). After contacting cultured cells with compounds

in microtitration plates, this method can measure residual viability of the cells by, e.g., [^{3}H] leucine incorporation followed by scintillation counting or by [^{35}S] methionine incorporation and autofluorography in situ. This is a method well known and routinely practiced in the art for assessing effects of chemical or physical perturbations on cultured cells including glioma cells. See, e.g., Oztopcu et al., Acta Neurol Belg. 104:106-10, 2004; Darling et al., Anticancer Drugs. 11:243-8, 2000; Sankar et al., Anticancer Drugs. 10:179-85, 1999; and Chen et al., Chin Med J (Engl). 107:808-12, 1994.

[0065] Other than using cultured glioma cells to screen for glioma inhibiting compounds in vitro, in vivo screening systems employing a glioma tumor animal model can also be used in the practice of the present invention. For example, Lumniczky et al., (Cancer Gene Ther. 9:44-52, 2002) developed a mouse brain tumor model to study treatment of glioma by chemotherapy and radiation therapy. Brain tumors were induced by intracranial injection of cultured Gl261 cells. This glioma animal model and other similar animal model systems known in the art can all be employed to examine the modulating compounds for ability to inhibit glioma growth.

VIII. Pharmaceutical Compositions

[0066] The glioma-inhibiting compounds described above can be directly administered under sterile conditions to the subject to be treated. The modulators can be administered alone or as the active ingredient of a pharmaceutical composition. The therapeutic composition of the present invention can also be combined with or used in association with other therapeutic agents. In some applications, a first glioma-inhibiting compound is used in combination with a second glioma-inhibiting compound in order to inhibit glioma growth to a more extensive degree than cannot be achieved when one glioma-inhibiting compound is used individually. In some other applications, a glioma-inhibiting compound of the present invention may be used in conjunction with known anti-glioma drugs such as procarbazine, lomustine, vincristine, and temozolomide (see, e.g., Papagikos et al., Lancet Oncol. 6:240-4, 2005; and Chang et al., 100:605-11, 2004).

[0067] Pharmaceutical compositions of the present invention typically comprise at least one active ingredient together with one or more acceptable carriers thereof. Pharmaceutically acceptable carriers enhance or stabilize the composition, or facilitate

preparation of the composition. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (*e.g.*, nucleic acid, protein, or modulatory compounds), as well as by the particular method used to administer the composition. They should also be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the subject. This carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral, sublingual, rectal, nasal, intravenous, or parenteral. For example, the glioma-inhibiting compound can be complexed with carrier proteins such as ovalbumin or serum albumin prior to their administration in order to enhance stability or pharmacological properties.

[0068] The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, capsules, and the like. The concentration of therapeutically active compound in the formulation may vary from about 0.1 100% by weight. Therapeutic formulations are prepared by any methods well known in the art of pharmacy. The therapeutic formulations can be delivered by any effective means which could be used for treatment. See, e.g., *Goodman & Gilman's The Pharmacological Bases of Therapeutics*, Hardman et al., eds., McGraw-Hill Professional (10^{th} ed., 2001); *Remington: The Science and Practice of Pharmacy*, Gennaro, ed., Lippincott Williams & Wilkins (20^{th} ed., 2003); and *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Ansel et al. (eds.), Lippincott Williams & Wilkins (7^{th} ed., 1999).

[0069] The therapeutic formulations can be conveniently presented in unit dosage form and administered in a suitable therapeutic dose. A suitable therapeutic dose can be determined by any of the well known methods such as clinical studies on mammalian species to determine maximum tolerable dose and on normal human subjects to determine safe dosage. Except under certain circumstances when higher dosages may be required, the preferred dosage of a glioma-inhibiting compound usually lies within the range of from about 0.001 to about 1000 mg, more usually from about 0.01 to about 500 mg per day.

[0070] The preferred dosage and mode of administration of a glioma-inhibiting compound can vary for different subjects, depending upon factors that can be individually reviewed by the treating physician, such as the condition or conditions to be treated, the

choice of composition to be administered, including the particular glioma-inhibiting compound, the age, weight, and response of the individual subject, the severity of the subject's symptoms, and the chosen route of administration. As a general rule, the quantity of a glioma-inhibiting compound administered is the smallest dosage which effectively and reliably prevents or minimizes the conditions of the subjects. Therefore, the above dosage ranges are intended to provide general guidance and support for the teachings herein, but are not intended to limit the scope of the invention.

EXAMPLES

[0071] The following examples are provided to illustrate, but not to limit the present invention.

Example 1. Identification of positive growth regulators of glioma by siRNA screening

[0072] In order to find novel genes involved in glioma growth, we screened of a focused siRNA library directed against 5000 genes that have the most potential to be druggable targets, with each gene represented by two different siRNAs (a total 10,000 siRNAs). A brain-derived glioblastoma cell line (A172 from ATCC) was employed in the screening. The siRNAs were spotted onto 384 well plates, with duplicate plate for each siRNA. siRNAs targeting EGFR8 and STAT3 were used as positive controls, while scramble siRNA was used as negative controls. Transfection of the cells with the siRNAs was initiated by applying Lipo2000/Opti-MEM mixture to each well. Following incubation at room temperature, A172 cells were added to the wells. The wells were incubated at 37°C CO_2 incubator for 4 days. Effects of the siRNAs on the growth of the glioma cells were examined by determining the number of viable cells with the CellTiter-Glo luminescent cell viability assay (Promega). Specifically, following addition of assay reagents to the wells, viable cells in each well is quantified by measuring relative luminescence in the wells using an Aquest Plate Reader.

[0073] The entire screen was conducted in duplicate, and plate data were normalized to a mean value and compared across the library by in house statistical software analysis. Data for each siRNA was compared to the mean signal of the entire plate, and expressed as the ratio afa/mfa, which is the average fold activation (afa) divided by the

adjusted standard deviation of the fold activation (mfa). The mfa penalizes the value for fold activation if the standard deviation between the replicates is high. Negative numbers represent fold of inhibition of growth due to siRNA knockdown of genes that play a positive role in the growth of the glioma cells. These genes are termed herein positive growth regulators of glioma.

[0074] As shown in Table 1, the screening yielded a number of hits whose knockdown by the siRNAs led to at least 2 folds of inhibition relative to the background average. 15 of the hits from the primary screening which displayed a preference for A172 cells over normal human astrocytes (NHA) were chosen for reconfirmation and validation. To eliminate off target effects, these hits were examined for their differential effects on gliomas cells (A172, U87, LN18) and NHA cells with additional multiple siRNAs against these hits. Each siRNA set (targeting the same gene) has 1 smartpool and 4 individuals. These addition siRNAs were examined on A172, U87, LN18 gliomas cells and NHA cells. These experiments were designed to reconfirm hits that (i) have at least the smartpool and 2 individuals siRNAs inhibit one gliomas cell growth; and (ii) whose knockdown by the siRNAs are less toxic on NHA than on gliomas cells. By these criteria, six of the 15 hits (CDC25B, NEK9, KIAA0703, ABL1, ING4 and CBLC) are reconfirmed as glioma growth enhancing genes.

[0075] The 6 reconfirmed hits were further subject to in vitro validation. As assessed by RT-quantitative PCR analysis, there was more than 90% CDC25B mRNA expression inhibition when treating the A172 cells with smartpool and one individual siRNAs targeting CDC25B. The expression inhibition is relative to expression level in control A172 cells that were treated with scramble siRNA (control). In addition, there was about 70% NEK9 mRNA expression inhibition when treating the A172 cells with smartpool and one individual siRNAs compared with the control. 70% KIAA mRNA expression inhibition was also achieved in A172 cells treated with one individual siRNA 2 days relative to the control. As examined by western blot analysis, CDC25B protein expression was almost completely blocked in A172 cells treated with smartpool and 2 individual siRNAs targeting CDC25B as compared to the control cells.

[0076] We also examined expression level of CDC25B with DNA array gene chip. The data indicate that CDC25B expression level in glioma cells are about 3 folds higher than that in NHA cells.

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[0077] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

[0078] All publications, GenBank sequences, ATCC deposits, patents and patent applications cited herein are hereby expressly incorporated by reference in their entirety and for all purposes as if each is individually so denoted.

WE CLAIM:

1. A method of inhibiting or ameliorating growth of a glioma tumor cell, the method comprising contacting the glioma tumor cell with an agent which down-regulates expression or cellular level of a positive growth regulator of glioma encoded by a polynucleotide selected from the members listed in Table 1; thereby inhibiting or ameliorating growth of the glioma tumor cell.

2. The method of claim 1, wherein the positive growth regulator of glioma is selected from the group consisting of CDC25B, NEK9, KIAA0703, ABL1, ING4 and CBLC.

3. The method of claim 1, wherein the agent is a short interfering RNA (siRNA) that specifically targets the positive growth regulator of glioma.

4. The method of claim 1, wherein the agent is selected from the group consisting of a microRNA (miRNA), a synthetic hairpin RNA (shRNA), an anti-sense nucleic acid, and a complementary DNA (cDNA).

5. The method of claim 1, wherein the glioma tumor cell is present in a subject.

6. The method of claim 5, wherein the subject is a human.

7. The method of claim 5, wherein the subject is administered a pharmaceutical composition comprising an effective amount of the agent.

8. A method of inhibiting or ameliorating growth of a glioma tumor cell, the method comprising contacting the glioma tumor cell with an agent which down-regulates a biological activity of a positive growth regulator of glioma encoded by a polynucleotide selected from the members listed in Table 1; thereby inhibiting or ameliorating growth of the glioma tumor cell.

9. The method of claim 8, wherein the positive growth regulator of glioma is an enzyme, and the biological activity is its enzymatic activity

10. The method of claim 8, wherein the positive growth regulator of glioma is selected from the group consisting of CDC25B, NEK9, KIAA0703, ABL1, ING4 and CBLC.

11. The method of claim 8, wherein the agent is an antagonist antibody that specifically binds to the positive growth regulator of glioma.

12. The method of claim 8, wherein the glioma tumor cell is present in a subject.

13. The method of claim 12, wherein the subject is a human

14. The method of claim 12, wherein the subject is administered a pharmaceutical composition comprising an effective amount of the agent.

15. A method for identifying an agent that inhibits glioma growth, the method comprising:

(a) screening test compounds to identify one or more modulating compounds that down-regulate a biological activity or expression of a positive growth regulator of glioma encoded by a polynucleotide selected from the members listed in Table 1; and

(b) testing the modulating compounds for ability to inhibit glioma growth.

16. The method of claim 15, wherein the positive growth regulator of glioma is selected from the group consisting of CDC25B, NEK9, KIAA0703, ABL1, ING4 and CBLC.

17. The method of claim 15, wherein the ability to inhibit glioma growth by the modulating compounds is examined by monitoring viability of a cultured glioma cell contacted with the compounds.

18. The method of claim 17, wherein the glioma cell is A172 glioma cell.

19. The method of claim 17, wherein viability of the glioma cell is monitored with CellTiter-Glo luminescent cell viability assay.

20. The method of claim 17, wherein the ability to inhibit glioma growth by the modulating compounds is determined by comparing viability of the glioma cell that has been contacted with a modulating compound with viability of a control glioma cell that has not been contacted with the compound.