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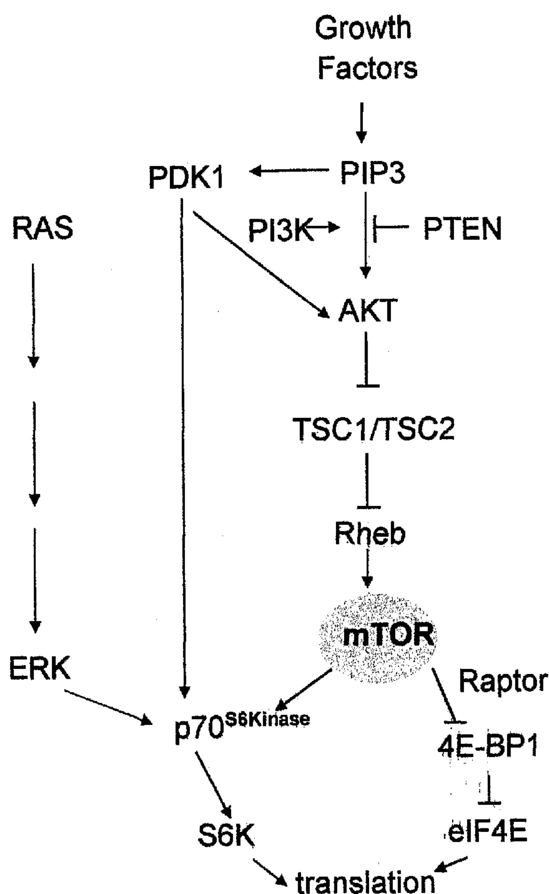
(54) Title: METHODS INVOLVING THE PI3K/AKT IN GLIOMAS AND PROSTATE CANCERS



(57) Abstract: The invention disclosed herein provides methods for the examination and/or quantification of the PI3K/Akt and Ras/MAPK biochemical pathways that are disregulated in pathologies such as cancers of the prostate and gliomas. The invention disclosed herein further provides reagents and kits adapted for performing such methods.

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METHODS INVOLVING THE PI3K/AKT IN GLIOMAS AND PROSTATE CANCERS

STATEMENT OF GOVERNMENT SUPPORT

[0001] This invention was made with support from U01 CA88127 from the National Cancer Institute/NIH and K08NS43147-01 from the National Institute of Neurological Disorders and Stroke/NIH. The government may have certain rights to 5 this invention.

RELATED APPLICATIONS

[0002] This application is a continuation-in-part of U.S. Patent Application Serial No. 10/701,490 filed November 5, 2003, which claims the benefit of U.S. 10 Provisional Application Serial No. 60/423,777 filed November 5, 2002, the contents of each of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0003] The present invention provides methods for the examination of 15 biochemical pathways that are shown to be disregulated in pathologies such as cancer and to reagents adapted for performing these methods.

BACKGROUND OF THE INVENTION

[0004] Cancers are the second most prevalent cause of death in the United 20 States, causing 450,000 deaths per year. One in three Americans will develop cancer, and one in five will die of cancer. While substantial progress has been made in identifying some of the likely environmental and hereditary causes of cancer, there is a need for additional diagnostic and therapeutic modalities that target cancer and related diseases and disorders. In particular, there is for a need a greater understanding of the various 25 biochemical pathways that are involved in disregulated cell growth such as cancer as this will allow for the development of improved diagnostic and therapeutic methods for identifying and treating pathological syndromes associated with such growth disregulation.

[0005] Biochemical pathways that are of particular interest in pathologies such as cancer are the PI3K/Akt and Ras/MAPK pathways. Specifically, deregulation of the PI3K/Akt and Ras/MAPK pathways occurs in many types of cancer (see, e.g., Vivanco et al., Nat Rev Cancer. *2:* 489-501., 2002), including glioblastoma (GBM) and cancer of the prostate (see, e.g., Vivanco et al., Nat Rev Cancer. 2: 489-501, 2002; Feldkamp et al., Journal of Neurooncology 35: 223-248, 1997; Mischel et al., Brain Pathology, Jan;13(1):52-61 2003). Because constitutively activated signal transduction cascades directly modulate biological behavior, and because new molecular approaches to cancer therapy focus on inhibiting these pathways (see, e.g., Sawyers et al., Curr Opin Genet Dev. 12: 111-5, 2002; Druker et al., Cancer Cell. 1: 31-6., 2002; Kilic et al., Cancer Res. 60: 5143-50, 2000; Neshat et al., Proc Natl Acad Sci U S A. 98: 10314-9, 2001), it is critical that they be detected in patient biopsies. Traditionally, biochemical approaches such as Western blots and *in vitro* kinase assays have been required to assess activation of these pathways (see, e.g., Neshat et al., Proc Natl Acad Sci U S A. *98:* 10314-9, 2001; Ermoian et al., Clin Cancer Res. *8:* 1100-6., 2002). However, these techniques are not feasible on routinely processed tissues such as formalin-fixed, paraffin-embedded patient biopsy samples. Currently, the tools to identify activation pathways in patient biopsy material have not been fully developed. Development of such tools is critical to determine whether these pathway activations have prognostic significance, and to help stratify patients for targeted molecular therapy.

[0006] Glioblastoma multiforme (GBM), the most common malignant brain tumor of adults (and one of the most lethal of all cancers) is highly suited for this approach. GBMs have a set of defined molecular lesions with resultant signaling pathway disruptions. The tumor suppressor gene PTEN is altered in 30-40% of GBMs (see, e.g., Liu et al., Cancer Res. *57:* 5254-7., 1997; Schmidt et al., J Neuropathol Exp Neurol. *58:* 1170-83., 1999; Smith et al., J Natl Cancer Inst. *93:* 1246-56., 2001). Since the PTEN lipid phosphatase activity negatively regulates activation of the Akt pathway and its downstream effectors mTOR, FKHR and S6 (see, e.g., Vivanco et al., Nat Rev Cancer. *2:* 489-501., 2002), it is possible that PTEN protein deficient GBMs would show coordinated activation of this pathway. Primary GBMs (those that arise as de novo grade

IV tumors) also commonly over-express the oncogene EGFR, and its variant EGFRvIII, which activate signaling through both the RAS/MAPK and PI3K/Akt pathways. Therefore, it is also possible that EGFR and EGFRvIII expressing GBMs would show coordinate activation of the ERK and the Akt pathways. To date however, the relationship between these various pathways has not been delineated.

[0007] Inactivation of the PTEN tumor suppressor gene is also implicated in the development of both primary and metastatic prostate cancers. Conditional deletions of PTEN in the mouse prostate leads to cancer and many human prostate cancer cell lines and xenografts have PTEN deletions or point mutations (see, e.g. Wang et al., Cancer Cell, 4: 209-221, 2003; Trotman et al., PLoS Biol, 1: E59, 2003; Steck et al., Nat Genet, 15: 356-362, 1997; Pesche et al., Oncogene, 16: 2879-2883, 1998; and Teng et al., Cancer Res, 57: 5221-5225, 1997). The best characterized function of PTEN is as a lipid phosphatase that counteracts the growth and survival promoting effects of phosphatidyl inositol 3-kinase (PI3K). PI3K is a lipid kinase that phosphorylates phosphatidylinositols at the 3-position (PtdIns [3, 4, 5] P_3), which subsequently recruit kinases such as AKT (a potent oncogenic survival factor), leading to a cascade of constitutive activation of downstream effectors (Figure 4), including the mammalian Target Of Rapamycin (mTOR) (Vivanco et al.,. Nat Rev Cancer, 2: 489-501, 2002; Hidalgo et al., Oncogene, 19: 6680-6686, 2000; Sawyers et al., Cancer Cell, 4: 343-348, 2003; Fingar et al., Oncogene, 23: 3151-3171, 2004; Luo et al., Cancer Cell, 4: 257-262, 2003).

[0008] While researchers have identified a variety of genes and pathways involved in pathologies such as cancer, there is need in the art for additional tools to facilitate the analyses of the regulatory processes that are involved in disregulated cell growth. Moreover, an understanding of how the products of genes involved in disregulated cell growth interact in a larger context is needed for the development of improved diagnostic and therapeutic methods for identifying and treating pathological syndromes associated with growth disregulation. In particular, there remains a need to identify signal transduction events driving oncogenesis and to identify markers useful for assessing progression or inhibition of the oncogenic phenotype. The methods and reagents disclosed herein satisfy this need.

SUMMARY OF THE INVENTION

[0009] Deregulated activation of the PI3K/Akt pathway is common in a variety of different cancers including glioblastomas and cancers of the prostate, bile duct, bladder, breast, colon, endometrium, blood, liver, lung, skin, ovary, pancreas and thyroid to name a few. Consequently, the assessment of this pathway can be used to obtain diagnostic, prognostic and therapeutic information, for example to stratify patients for targeted kinase inhibitor therapy. The disclosure provided herein identifies a series of biomarkers that are associated with deregulated activation of the PI3K/Akt pathway as well as optimized methods for examining these markers. Significantly, the disclosed methods for examining these markers are useful with a wide variety of tissue samples including formalin fixed, paraffin embedded biopsy samples.

[0010] As disclosed herein, a series of PI3K/Akt pathway biomarkers associated with cancers such as prostate cancer and glioblastoma multiforme can be examined using for example a series of antibodies such as phospho-specific antibodies. In typical methods, a mammalian cell such as a cancer cell derived from a biopsy sample can be examined for evidence of PI3K/Akt pathway activation by examining a tissue sample containing this cell for the presence of: a phosphorylated S6 polypeptide (SEQ ID NO: 1); a phosphorylated mTOR polypeptide (SEQ ID NO: 2); a phosphorylated FKHR polypeptide (SEQ ID NO: 3); a phosphorylated AKT polypeptide (SEQ ID NO: 4); a phosphorylated ERK polypeptide (SEQ ID NO: 8); or decreased levels of expression of the PTEN polypeptide (SEQ ID NO: 5), wherein the presence of a phosphorylated S6, mTOR, FKHR, AKT or ERK polypeptide, or decreased levels of expression of the PTEN polypeptide, provides evidence of Akt pathway activation in the cell. Optionally the cell is examined for the presence of a plurality of characteristics such as a phosphorylated S6 polypeptide (SEQ ID NO: 1) and decreased levels of expression of the PTEN polypeptide (SEQ ID NO: 5). Certain embodiments of the invention comprise further methodological steps, the step of using the results of the examination to identify and/or assess a therapeutic agent that may be used to treat the cancer such as the step of using the results of the examination to evaluate the effect of an mTOR inhibitor

such as rapamycin or an analogue thereof or an EGFR inhibitor such as ZD-1839 or an analogue thereof on a cancer cell.

[0011] One preferred embodiment of the invention is a method for identifying a mammalian glioma tumor likely to respond or responsive to an EGFR polypeptide (SEQ ID NO: 7) inhibitor or an mTOR polypeptide (SEQ ID NO: 2) inhibitor, the method comprising examining a sample obtained from the tumor for: the expression of PTEN polypeptide (SEQ ID NO: 5); and the presence of at least one of, a phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1); a EGFR polypeptide (SEQ ID NO: 7); a phosphorylated AKT polypeptide (SEQ ID NO: 4); and a phosphorylated ERK polypeptide (SEQ ID NO: 8), wherein decreased expression of PTEN polypeptide together with decreased phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the glioma tumor as likely to respond or responsive to an mTOR inhibitor, and wherein decreased expression an of PTEN together with normal phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the glioma tumor as not likely to respond or non-responsive to an mTOR inhibitor, and wherein normal or increased expression of PTEN and increased expression and/or activity of EGFR together with increased phosphorylation of AKT and/or phosphorylation of ERK identifies the glioma tumor as not likely to respond and/or non-responsive to an EGFR inhibitor.

[0012] Another preferred embodiment of the invention is a method for identifying a mammalian prostate tumor likely to respond or responsive to an EGFR polypeptide (SEQ ID NO: 7) inhibitor or an mTOR polypeptide (SEQ ID NO: 2) inhibitor, the method comprising examining a sample obtained from the tumor for: the expression of PTEN polypeptide (SEQ ID NO: 5); and the presence of at least one of, a phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1); a EGFR polypeptide (SEQ ID NO: 7); a phosphorylated AKT polypeptide (SEQ ID NO: 4); and a phosphorylated ERK polypeptide (SEQ ID NO: 8), wherein decreased expression of PTEN polypeptide together with decreased phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the prostate tumor as likely to respond or responsive to an mTOR inhibitor, and wherein decreased expression an of PTEN together with

normal phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the prostate tumor as not likely to respond or non-responsive to an mTOR inhibitor, and wherein normal or increased expression of PTEN and increased expression and/or activity of EGFR together with increased phosphorylation of AKT and/or phosphorylation of ERK identifies the prostate tumor as not likely to respond and/or non-responsive to an EGFR inhibitor.

[0013] Another embodiment of the invention is a kit for characterizing a mammalian tumor or cell, the kit comprising: an antibody that binds PTEN (SEQ ID NO: 5); and/or an antibody that binds phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1); and/or an antibody that binds EFGR (SEQ ID NO: 7); and/or an antibody that binds phosphorylated AKT (SEQ ID NO: 4); and/or an antibody that binds phosphorylated ERK (SEQ ID NO: 8). Optionally the kit further includes an antibody that binds Ki-67 polypeptide (SEQ ID NO: 9), and/or p-H3 histone polypeptide (SEQ ID NO: 10) and/or caspase-3 polypeptide (SEQ ID NO: 11). Typically the kit further comprises a secondary antibody which binds to one of the primary antibodies directed to these polypeptides. Optionally the kit comprises a plurality of antibodies that bind to the various polypeptides.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 shows the immunohistochemical expression of PTEN, p-Akt, p-mTOR, p-FKHR and p-S6 in GBM tumor samples. (A) Representative images demonstrating PTEN protein loss in tumors cells with retention of staining in vascular endothelium (0), diminished PTEN staining relative to the endothelium (1), and no evidence of PTEN protein loss (2). NC is the negative control. (B) Staining for p-Akt, p-mTOR, p-FKHR and p-S6 scored on a scale of 2 (strong), 1 (mild) and 0 (negative). NC represents negative controls.

[0015] Figure 2 shows the immunohistochemical expression of EGFR, EGFRvIII and p-Erk in GBM tumor samples. (A) Representative images demonstrating diffuse EGFR, EGFRvIII and p-Erk positivity (+). Representative images of tumors

lacking EGFR, EGFRvIII and p-ERK expression are also shown (-). NC represents the negative controls.

[0016] Figures 3A and 3B provide an illustration of the interaction between members of the PI3K/Akt pathway and kinase inhibitors in GBM tumor samples. Figure 3A shows that rapamycin inhibits S6 phosphorylation in glioblastoma in vivo. Figure 3B shows that the rapamycin-mediated inhibition of S6 phosphorylation correlates with diminished tumor proliferation. In this Figure, Ki-67, a marker of cellular proliferation was used to assess whether rapamycin-mediated inhibition of S6 had an effect on tumor growth.

[0017] Figure 4 provides a schematic demonstrating the components of the PI3K signaling pathway.

[0018] Figures 5A-5E provide a validation of PTEN and the phospho-specific antibodies. **A)** PTEN expression in prostate cancer cell lines. Sections of cell pellets prepared from DU145 (PTEN Wild Type) and LNCaP (Deletion of one allele and mutation in second allele). Note the lack of immunostaining in LNCaP cells. **B)** p-AKT, p-S6, p-FKHR and p-ERK immunostaining in a formalin fixed, paraffin embedded breast tumor section. The left panel shows positive staining with the antibody. There is no staining when a specific antigen peptide is used in combination with the respective phospho-specific antibodies, right panel. **C)** Immunofluorescent detection of total AKT (red) or p-AKT Ser473(green) in paraffin-embedded LNCaP cells, untreated (left) or treated with 30μM LY294002 (right). Yellow represents overlay. Note the decreased intensity of phosphorylated AKT staining in the LY294002-treated cells, with unchanged expression of AKT. **D)** LNCaP cells were grown in culture and exposed either to vehicle or the PI3K inhibitor, LY294002 (30μM), for18 hours. Cell pellets were prepared, formalin fixed and embedded in paraffin, then cut for immunostaining with phospho-AKT, p-FKHR, p-S6 and p-ERK antibodies. The addition of LY 2940002 resulted in decreased staining intensity of p-AKT, p-FKHR and p-S6K. The staining intensity of p-ERK before and after treatment remained unchanged. Next, p-ERK staining was examined in HT129 cells, by the addition of EGF (100ng/ml for 20 minutes). This staining is subsequently reduced when a MEK inhibitor, UO-126 (20μM for 20 minutes)

is added to the induced cells, in contrast to lack of inhibition by LY 294002. **E)**. LNCaP cells were grown in culture and exposed to either vehicle or the mTOR inhibitor, CCI-779 (20nM) for 18 hours and subsequently prepared as described in the material and methods section. Sections were stained with p-AKT and p-S6. The addition of CCI-779 resulted in decreased staining intensity of p-S6 but had no effect on p-AKT, which is consistent with mTOR being downstream of AKT but upstream of p-S6.

[0019] Figures 6A and 6B provide a definition of PTEN scoring criteria. **A)** Range of PTEN immunostaining. In our series, PTEN protein expression was found to be absent in 29% (Score=0, a), decreased compared to benign glands in 11% (Score=1, b) and equivalent to benign glands in 60% of tumors (Score=2, c). Arrows indicate staining characteristics. **B)** PI3K pathway activation in a patient with loss of PTEN expression. Serial sections from the same tumor show loss of PTEN expression results in the phosphorylation of p-AKT, p- S6 and p-FKHR. In this case, p-ERK is also active.

[0020] Figure 7 provides a demonstration of the inter-relationships between signaling molecules *in vivo*. MDS analysis shows that PTEN protein and AKT phosphorylation are tightly linked, confirming what is known of their *in vitro* relationship.

DETAILED DESCRIPTION OF THE INVENTION

[0021] Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995). As appropriate, procedures involving the use of commercially

available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

[0022] "Mammal" for purposes of treatment or therapy refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

[0023] The terms "cancer", “cancerous", or “malignant” refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to astrocytoma, blastoma, carcinoma, glioblastoma, leukemia, lymphoma and sarcoma. More particular examples of such cancers include breast cancer, ovarian cancer, colon cancer, colorectal cancer, rectal cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, Hodgkin’s and non-Hodgkin’s lymphoma, testicular cancer, esophageal cancer, gastrointestinal cancer, renal cancer, pancreatic cancer, glioblastoma, cervical cancer, glioma, liver cancer, bladder cancer, hepatoma, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

[0024] "Growth inhibition" when used herein refers to the growth inhibition of a cell *in vitro* and/or *in vivo*. The inhibition of cell growth can be measured by a wide variety of methods known in the art. A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell *in vitro* and/or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Such agents further include inhibitors of cellular pathways associated with disregulated cell growth such as the PI3K/Akt pathway. Further information can be found in The

Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami *et al.* (WB Saunders: Philadelphia, 1995).

[0025] "Treatment" or "therapy" refer to both therapeutic treatment and prophylactic or preventative measures. The term "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy *in vivo* can, for example, be measured by assessing tumor burden or volume, the time to disease progression (TTP) and/or determining the response rates (RR).

[0026] The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies and antibody compositions with polyepitopic specificity (e.g. polyclonal antibodies) as well as antibody fragments so long as retain their ability to immunospecifically recognize a target polypeptide epitope.

[0027] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially

homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., Nature, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature, 352:624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991), for example.

[0028] As used herein, the term "polynucleotide" means a polymeric form of nucleotides of at least 10 bases or base pairs in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide, and is meant to include single and double stranded forms of DNA and/or RNA. In the art, this term if often used interchangeably with "oligonucleotide". A polynucleotide can comprise a nucleotide sequence disclosed herein wherein thymidine (T) can also be uracil (U); this definition pertains to the differences between the chemical structures of DNA and RNA, in particular the observation that one of the four major bases in RNA is uracil (U) instead of thymidine (T).

[0029] As used herein, the term "polypeptide" means a polymer of at least about 10 amino acids. Throughout the specification, standard three letter or single letter designations for amino acids are used. In the art, this term is often used interchangeably with "protein".

[0030] As used herein, the term "inhibitor" encompasses molecules capable of inhibiting one or more of the biological activities of target molecules such as mTOR and/or EGFR polypeptide. Illustrative inhibitors include the targeted small-molecule inhibitors and antibody inhibitors disclosed herein as well as other inhibitors known in the art such as anti-sense polynucleotides and siRNA. Consequently one skilled in the art will appreciate that such inhibitors encompass molecules which inhibit both polynucleotide synthesis and/or function (e.g. antisense polynucleotide molecules) as well those which inhibit polypeptide synthesis and/or function (e.g. molecules which block phosphorylation and hence activity of a target polypeptide such as mTOR).

Physiological Processes Pertinent To The Invention

[0031] Deregulated activation of the PI3K/Akt pathway is common in a variety of different cancers (see, e.g. Fresno Vara et al., Cancer Treat Rev.. 30(2): 193-204 (2004; Mitsiades et al., Curr. Cancer Drug targets, 4(3): 235-256 (2004); Brader et al., Tumori, 90(1): 2-8 (2004); and Sansal et al., J. Clin. Oncol., 22(14): 2954-2963 (2004). An illustrative but non limiting list of cancers that exhibit deregulated activation of the PI3K/Akt pathway, include glioblastomas and cancers of the prostate (see, e.g., Vivanco et al., Nat Rev Cancer. 2: 489-501, 2002; Feldkamp et al., Journal of Neurooncology 35: 223-248, 1997; Mischel et al., Brain Pathology, Jan;13(1):52-61 2003) as well as cancers of the bile duct (see, e.g. Tanno et al., Cancer Res., 64(10): 3486-3490 (2004)), bladder (see, e.g. Riegler-Christ et al., Oncogene, 23(27): 4745-53 (2004), breast (see, e.g. DeGraffenried et al., Ann. Oncol., 15(10): 1510-1516 (2004)), colon (see, e.g. Itoh et al., Cancer, 94(12): 3127-34 (2004)), endometrium (see, e.g. Gagnon et al., Int. J. Oncol., 23(2): 803-10 (2003), leukocytes (see, e.g. Cuni et al., Leukemia, 18(8): 1438-40 (2004); Kubota et al., Leukemia, 18(8): 1391-400 (2004); and Tabelli et al., Br. J. Haematol. 126(4): 574-82 (2004)), liver (see, e.g. Wang et al., Genes Devel. 18(8): 912-25 (2004)), lung (see, e.g. Sithanandam et al., Carcinogenesis 24(10): 1581-92 (2003); and Cappuzzo et al., J. Natl. Cancer Inst. 96(15): 1133-1141 (2004)), melanocytes (see, e.g. Dhawan et al., 62(24): 7335-42 (2002)) ovary (see, e.g. Altomare et al., Oncogene 23(24): 5853-7 (2004)) pancreas (see, e.g. Perugini et al., J. Surg. Res. 90(1): 39-44 (2000) and thyroid (see, e.g. Vasko et al., J. Med. Genet. 41(3): 161-70 (2004)). Consequently, the assessment of this pathway is critical for stratifying patients for targeted kinase inhibitor therapy. The disclosure provided herein identifies a series of biomarkers that are associated with deregulated activation of the PI3K/Akt pathway as well as optimized methods for examining these markers. Consequently, the disclosure provided herein allows the examination of this pathway in cancer. Significantly, the disclosed methods for examining these markers are useful with a wide variety of tissue samples including formalin fixed, paraffin embedded biopsy samples. Various aspects of this disclosure are described in Choe et al., Cancer Res. 2003 Jun 1;63(11):2742-6.

[0032] As noted above, the disclosure provided herein identifies a series of biomarkers that are associated with deregulated activation of the PI3K/Akt pathway, a pathway whose deregulated activation is common in cancers such as gliomas. The disclosure provided herein further provides optimized methods for examining these biomarkers. Consequently, the disclosure allows the examination of the activation status of these biomarkers in a variety of cancers. As the art that teaches that this growth related pathway is common pathway that is disregulated in a wide variety of human cancers, artisans understand that the methods and materials disclosed herein can be universally applied to examine this pathway in all cancers in which the deregulated activation of the PI3K/Akt pathway is observed.

[0033] Significantly, the disclosed methods for examining these biomarkers are useful with a wide variety of tissue samples including formalin fixed, paraffin embedded biopsy samples. As disclosed herein, these markers can be examined using a panel of antibodies such as phospho-specific antibodies. In these methods, a mammalian cell such as a cell derived from a formalin fixed, paraffin embedded glioblastoma multiforme biopsy sample can be examined for evidence of Akt pathway activation by examining a tissue sample containing this cell for the presence of the various target molecules disclosed herein including phosphorylated polypeptides. Certain embodiments of the invention identify and/or assess a therapeutic agent that may be used to treat the glioblastoma such as rapamycin or an analogue thereof or an EGFR inhibitor such as ZD-1839 or an analogue thereof.

[0034] As noted above, the invention disclosed herein provides methods and immunohistochemical reagents that can be used to identify the activation state of the PI3K/Akt signaling pathway in clinical samples such as prostate and glioblastoma biopsy samples. These methods and reagents identify a coordinate regulation of the Akt/mTOR signaling pathway in response to loss of the PTEN tumor suppressor gene. As specific kinase inhibitors that target this pathway are currently in development (see, e.g., Neshat et al., Proc Natl Acad Sci U S A. *98:* 10314-9, 2001), and further because this mutation is common in glioblastoma and prostate cancer, this disclosure provides an important clinical tool for selecting patients for appropriate therapy. In this context, the

invention can be practiced by performing immunohistochemical analysis on routinely processed patient biopsy samples. The results of these assays can be used as criteria for inclusion in clinical trials, and to assess outcome differences in patients in which this pathway is deregulated.

5 **[0035]** The methods and reagents disclosed herein can be used to determine the activation state of biomarker polypeptides such as Akt and its downstream effectors such as mTOR, ERK, Forkhead and S6-kinase on routinely processed patient biopsy samples (e.g. glioblastoma samples) and this information can be used to select patients for therapy with targeted pathway inhibitors. As disclosed herein, the invention has been tested on 10 biopsies from patients suffering from glioblastoma as well as patients suffering from cancers of the prostate. The results demonstrate clear coordinate regulation of Akt, mTOR, forkhead and S6-kinase, and their association with PTEN loss. A detailed discussion of the biomarkers and the physiological processes pertinent to the invention is provided below.

15 **[0036]** Activation of PI3K by growth factor signaling catalyzes the formation of phosphatidylinositol triphosphate (PIP3) by addition of a phosphate group to phosphoinositol bisphosphate (PIP2). PIP3 catalyzes the activation of the Akt kinase (and its downstream effectors mTOR, forkhead and S6-kinase), which promote cell proliferation and survival. The PTEN tumor suppressor gene encodes a phosphatase 20 that removes the phosphate group from PIP3, thereby regulating the activation state of this pathway. PTEN loss results in constitutive signaling through PIP3, and hence unregulated activation of the Akt pathway.

[0037] PTEN is lost in many types of cancer including glioblastomas and cancers of the prostate. In addition, the Akt pathway is dysregulated in many other 25 cancers. PTEN-deficient cancer cells are dramatically more sensitive to inhibition of the Akt pathway at the level of mTOR (see, e.g., Neshat et al., Proc Natl Acad Sci U S A. *98:* 10314-9, 2001), than PTEN wild-type cells, including non-cancerous cells. Therefore, mTOR inhibitors can be a highly selective and effective therapy for patients whose tumors have PTEN loss and Akt pathway activation. All prior knowledge of the 30 PTEN/PI3K/Akt pathway is based on biochemical data and genomic analysis, which are

not feasible as a clinical screening tool. Currently, there are no methods for detection of the activation state of this pathway in routinely processed formalin-fixed, paraffin-embedded patient biopsy samples. Consequently, the ability to identify the activation state of this pathway in such clinical samples, and to select patients for its inhibition is a
5 valuable diagnostic tool. This is also valuable tool for the analyses of inhibitors that target this pathway.

[0038] As specifically disclosed herein we demonstrate that PI3'K/Akt pathway activation can be detected in routinely processed patient biopsies. We demonstrate that PTEN loss is significantly correlated with Akt activation, which is significantly associated
10 with activation of downstream effectors mTOR, S6 and FKHR. We have also shown that PTEN loss is not the only mechanism of PI3'K/Akt pathway activation, and demonstrated that EGFR and EGFRvIII co-expression are significantly associated with activation of this pathway. Finally, we demonstrate that PI3K/Akt and Erk pathway activation have significant impact on cancer progression and survival. These data
15 provides evidence that this set of tools can be used to stratify cancer patients for targeted molecular therapy.

[0039] The epidermal growth receptor factor receptor contributes to the malignant phenotype of human glioblastomas (see, e.g. Thomas et al., Int J Cancer. 2003 Mar 10;104(1):19-27). Studies in SKMG-3 cells, a GBM cell line that maintains EGFR
20 gene amplification in vitro demonstrate that EGF treatment stimulated phosphorylation of the EGFR as well as the downstream effectors Erk, AKT1, stat3 and c-Cbl. Under minimal growth conditions, unstimulated SKMG-3 cells contain constitutively phosphorylated Erk and AKTI. The EGFR kinase inhibitor PD158780 reduces the constitutive phosphorylation of the receptor and Erk but not that of AKT1. In contrast,
25 inhibition of phosphatidylinositol-3-kinase (PI3K) blocks the constitutive phosphorylation of Erk and AKT-1 but not the EGFR. The results provide evidence that signals from overexpressed EGFR contribute to the constitutive phosphorylation of Erk, but these signals may not required for the constitutive activation of PI3K or AKT1. See, e.g. Thomas et al., Int J Cancer. 2003 Mar 10;104(1):19-27.

[0040] In addition, EGFR appears to play an important role in the pathogenesis of colorectal cancer as shown for example by studies of the EGFR tyrosine kinase inhibitor ZD1839 in metastatic colorectal cancer patients in which serial biopsies were taken pre- and posttreatment to assess biological activity (see, e.g. Daneshmand Clin Cancer Res. 2003 Jul;9(7):2457-64). In these studies, paired biopsies were obtained from colorectal cancer patients before and after treatment. Posttreatment samples showed a statistically significant reduction in cancer cell proliferation. While all pretreatment samples showed strong staining for EGFR, loss of immunohistochemical staining for activated EGFR, phosphorylated Akt, and phosphorylated ERK in cancer cells was observed in some patients after treatment. See e.g., Daneshmand Clin Cancer Res. 2003 Jul;9(7):2457-64.

[0041] The PI3'K/Akt pathway is commonly deregulated in GBMs, but its identification in routine biopsies has presented a challenge. In the face of new kinase inhibitors that target this pathway, the need for an assay that can be used to stratify patients for therapy has become critical. As disclosed herein, we demonstrate that activation of the PI3'K/Akt pathway can be detected by immunohistochemistry using a panel of phospho-specific antibodies. We show that 38% of untreated primary GBMs demonstrate evidence of PTEN protein loss, and that this is significantly associated with Akt activation. We further demonstrate that phosphorylation of Akt is significantly correlated with phosphorylation of downstream effectors mTOR, FKHR and S6. We show that PTEN loss is not the only mechanism underlying Akt pathway activation; phosphorylation of Akt, mTOR, S6 and FKHR are also associated with co-expression of EGFR and its constitutively active variant EGFRvIII. Finally, we demonstrate that activation of the PI3'K/Akt and Erk pathways is associated with shorter time to progression and diminished overall survival in GBM patients.

[0042] The disclosure provided herein demonstrates that PI3'K/Akt pathway activation can be detected in paraffin-embedded biopsy samples, and provides evidence that PTEN loss is highly correlated with Akt pathway activation in primary GBMs. These results also provide evidence that co-expression of EGFR and EGFRvIII can activate the PI3'K pathway in GBMs with normal PTEN immunohistochemical

expression. The results further provide evidence that activation of these signaling pathways has considerable impact on GBM patient progression and survival.

[0043] The disclosure provided herein specifically demonstrates that the activation of the PI3'K/Akt pathway can be detected with phospho-specific antibodies in routinely processed patient biopsies. In one illustrative embodiment of the invention, the disclosed methods and materials are used to examine glioblastomas. In another illustrative embodiment of the invention that is disclosed herein, the disclosed methods and materials are used to examine cancers of the prostate. In the context of glioblastomas, we show that PTEN-deficient GBMs have coordinated activation of the Akt pathway and its downstream effectors mTOR, FKHR and S6. We also show that GBMs co-expressing EGFR and EGFRvIII have activation of the PI3'K/Akt and Erk signaling pathways. Finally, we demonstrate that activation of these signal transduction pathways has prognostic importance. For example, primary GBM patients whose tumors are activated downstream of Akt, or at the level of ERK, have significantly shorter time to tumor progression and significantly diminished overall survival. These results define molecular subtypes of GBMs and may be used to stratify patients for targeted molecular therapy.

[0044] As disclosed in detail below, in illustrative analytical methods we generated a tissue microarray from 45 untreated primary GBM patient biopsies and analyzed the immunohistochemical expression of p-Akt and downstream effectors p-mTOR, p-FKHR and p-S6, as well as p-Erk. EGFR, EGFRvIII expression, and PTEN loss, all of which can promote activation of the PI3'K/Akt pathway, were also analyzed and association with PI3'K/Akt and Erk pathway activation were determined. The prognostic implications of PI3'K/Akt and Erk pathway activation were also analyzed.

[0045] In our analysis the loss of PTEN immunohistochemical expression was detected in 38% of GBMs. Diminished PTEN protein expression was significantly associated with phosphorylation of Akt ($p<0.00001$) and downstream effectors mTOR ($p=0.04$), FKHR ($p=0.006$) and S6 ($p=0.001$). PTEN protein loss was not associated with Erk activation, which is independent of PI3'K/Akt signaling. PTEN protein loss was not the only route to PI3'K/Akt pathway activation; co-expression of EGFR and

EGFRvIII was significantly correlated with expression of p-Akt (p=0.06), p-mTOR (p=0.001), p-FKHR (p=0.002) and p-S6 (p=0.001) in GBMs with normal PTEN protein expression. EGFR and EGFRvIII co-expression was also associated with Erk activation (p=0.007). Concurrent phosphorylation of mTOR, FKHR and S6, was significantly associated with shorter time to progression (p=0.002) and decreased overall survival (p=0.02), as was Erk activation (p=0.04).

[0046] As noted above, the methods disclosed herein typically employ immunohistochemical analysis. Immunohistochemical analysis requires a subjective determination by pathologists. Proteomic approaches have the potential to be a more objective and sensitive methods and may become clinically feasible in the future (see, e.g., Liotta et al., Jama. *286:* 2211-4., 2001; Liotta et al., Breast Cancer Res. *2:* 13-4, 2000; Petricoin et al., Lancet. *359:* 572-7., 2002; Petricoin et al., Nat Rev Drug Discov. *1:* 683-95., 2002). However, the current need to stratify patients for targeted therapy, and to assess molecular correlates of response to experimental targeted agents, dictates that we develop assays that work on routinely processed biopsy samples using currently accessible methods. Activated Akt can be detected by immunohistochemistry done on patient biopsies, and it has been suggested that it may have biological or prognostic implications (see, e.g., Gupta et al., Clin Cancer Res. *8:* 885-92., 2002; Malik et al., Clin Cancer Res. *8:* 1168-71., 2002). Complementary to previous studies, we demonstrate here that a panel of phospho-specific antibodies can be used to detect p-Akt and its downstream effectors in order to map PI3'K/Akt pathway activation. The high level of association between the downstream effector activation and Akt phosphorylation, provides evidence that we have accurately assessed this pathway. Further, our data showing that PI3'K/Akt pathway activation is associated with PTEN protein loss (see, e.g., Neshat et al., Proc Natl Acad Sci U S A. *98:* 10314-9., 2001; Ermoian et al., Clin Cancer Res. *8:* 1100-6., 2002) or EGFR/EGFRvIII signaling, are highly consistent with recent *in vitro* and in animal models (see, e.g., Davies et al., Cancer Res. *59:* 2551-6., 1999; Davies et al., Cancer Res. *58:* 5285-90., 1998; Lorimer et al., Biochim Biophys Acta. *1538:* 1-9., 2001; Moscatello et al., J Biol Chem. *273:* 200-6., 1998), including a recent biochemical demonstration that PTEN protein level is inversely correlated with Akt

activation in GBM patient biopsies (see, e.g., Ermoian et al., Clin Cancer Res. *8:* 1100-6., 2002).

[0047] Our finding that ERK and PI3'K/Akt pathway activation were associated with shorter time to progression and decreased overall survival is the first demonstration that pathway activation may have an impact on GBM patient prognosis. The data presented herein provides evidence that pathway activation status conveys important prognostic information. It is surprising that Akt activation was not significantly associated with progression or survival, while downstream activation at the level of mTOR, S6 and FKHR was. This result raises two possibilities. Either the p-Akt antibody is a less sensitive tool for detecting PI3'K/Akt pathway activation than is the panel of downstream phospho-specific antibodies. Alternatively, convergent inputs to mTOR, FKHR and S6 downstream of Akt, or in and Akt-independent fashion, may play an important role in modulating the biological behavior of GBMs. In line with this, concurrent Erk and Akt-mediated signaling may be required for optimal activation of p70 S6 kinase, and formation of p-S6 (see, e.g., Iijima et al., J Biol Chem. *277:* 23065-75., 2002; Shi et al., J Biol Chem. *277:* 15712-20., 2002). Similarly, Akt-independent mechanisms of mTOR and FKHR phosphorylation have been demonstrated (see, e.g., Gingras et al., Genes and Development. *15:* 807-826., 2001; Burgering et al., Trends Biochem Sci. *27:* 352-60., 2002). Using the disclosure provided herein and methods typically employed in the art one can determine whether these additional inputs play a role in modulating GBM behavior. For additional discussions of EGFR and Akt activity and inhibitors thereof, see, e.g. Bianco et al., Oncogene, 2003 May 8;22(18):2812-22; Yakes et al., Cancer Res. 2002 Jul 15;62(14):4132-41; and She et al., Clin Cancer Res. 2003 Oct 1;9(12):4340-6, the contents of which are incorporated herein by reference

[0048] While the sample size of 45 patients is relatively modest, it was large enough to provide robust associations between PTEN loss and PI3'K/Akt pathway activation. Only untreated primary GBM patients were included in this study. Since treatment itself may modulate Erk and PI3'K/Akt pathway activation, this study design enabled us to better assess the association between pathway activation and upstream molecular events. Using the disclosure provided herein and methods typically employed

in the art one can perform both retrospective, and prospective analyses of GBM patients (both treated and untreated) to further quantify the prognostic implications of pathway activation and to identify molecular correlates of response to therapy.

[0049] In order to address any subjectivity of immunohistochemical analysis all immunostains were interpreted independently by two neuropathologists, and by one of the neuropathologists at independent occasions, and the inter-rater and intra-rater agreement were high for all stains. This provides evidence that interpretation of these phospho-specific antibodies will be reproducible between independent pathologists. In the future, more objective methods such as proteomic analysis can replace these tools (see, e.g., Liotta et al., Jama. *286:* 2211-4., 2001; Petricoin et al., Nat Rev Drug Discov. *1:* 683-95., 2002). Nonetheless, the data presented here provides evidence that we can accurately assess these pathways using currently available methods, and provides evidence that one can stratify patients for therapy.

[0050] GBMs are among the most heterogeneous tumors, as has been previously shown (see, e.g., Cheng et al., J Neuropathol Exp Neurol. *58:* 120-8., 1999; Jung et al., J Neuropathol Exp Neurol. *58:* 993-9., 1999). This poses a problem for assessment of molecular alterations in GBMs, as well as for stratification of patients for targeted inhibitor therapy. Using the disclosure provided herein and methods typically employed in the art one can directly determine the extent of intra-tumor molecular heterogeneity for PTEN, EGFR and EGFRvIII and assess the impact of this on pathway activation, prognosis and response to therapy.

[0051] As noted above, typical embodiments of the invention examine cellular pathways in the family of tumors termed "gliomas". Briefly, the brain contains two major cell types: neurons and glia. Glial cells give rise to the family of tumors termed "gliomas". There are several distinct types of tumors within this glioma grouping. These can range from very benign, slow-growing tumors to rapidly enlarging, highly malignant cancerous types. The most commonly occurring tumors within the glioma family are astocytomas, oligodendroglioma and ependymomas. In addition, some patients may have tumors with a mixed appearance. Astrocytomas are the most common type of glioma. These are tumors that occur within the brain tissue itself. Like all gliomas,

astrocytomas can be located either superficially or deep within the brain and can affect critical structures. As they arise from the astrocyte cells (which serve as supporting elements of the brain), astrocytomas are generally infiltrative in nature.

[0052] As discussed in detail below, the World Health Organization (WHO) grading scheme is used to characterize this group of tumors. Briefly, in the World Health Organization grading system, grade I tumors are the least malignant. These tumors grow slowly and microscopically appear almost normal; surgery alone may be effective. Grade I tumors are often associated with long-term survival. Grade II tumors grow slightly faster than grade I tumors and have a slightly abnormal microscopic appearance. These tumors may invade surrounding normal tissue, and may recur as a grade II or higher tumor. Grade III tumors are malignant. These tumors contain actively reproducing abnormal cells and invade surrounding normal tissue. Grade III tumors frequently recur, often as grade IV tumors. Grade IV tumors are the most malignant and invade wide areas of surrounding normal tissue. These tumors reproduce rapidly, appear very unusual microscopically and are necrotic (have dead cells) in the center. Grade IV tumors cause new blood vessels to form, to help maintain their rapid growth. Glioblastoma multiforme is the most common grade IV tumor. For additional information see, e.g. Tatter SB , Wilson CB, Harsh GR IV. Neuroepithelial tumors of the adult brain. In Youmans JR, ed. Neurological Surgery, Fourth Edition, Vol. 4: Tumors. W.B. Saunders Co., Philadelphia, pp. 2612-2684, 1995; Kleihues P, Burger PC, Scheithauer BW. The new WHO classification of brain tumours. Brain Pathology 3:255-68, 1993; Lopes MBS, VandenBerg SR, Scheithauer BW; The World Health Organization classification of nervous system tumors in experimental neuro-oncology. In A.J. Levine and H.H. Schmidek, eds. Molecular Genetics of Nervous System Tumors Wiley-Liss, New York, pp. 1-36, 1993.

[0053] Low-grade astrocytomas (Grades I/IV or II/IV) are termed benign and occur generally in children or young adults. These tumors carry a better prognosis than higher grade astrocytomas. Although the management of these low-grade astrocytomas can be controversial, those tumors which are surgically accessible are usually resected. One of the concerns with low-grade astrocytomas in adults is that they can undergo a

malignant transformation and change into a higher-grade, or malignant tumor. The methods of the invention can be used to monitor such transformations. In astrocytomas grade I, normal karyotype is observed most frequently; among the cases with abnormal karyotypes, the most frequent chromosomal abnormalities loss of the X and Y sex-chromosomes; loss of 22q is found in 20-30% of astrocytomas; other abnormalities observed in low grade tumors include gains on chromosome 8q, 10p, and 12p, and losses on chromosomes 1p, 4q, 9p, 11p 16p, 18 and 19.

[0054] Anaplastic astrocytomas (Grade III/IV) are more aggressive tumors and, as such, are usually treated in a more radical fashion. In anaplastic astrocytomas, chromosome gains or losses are frequent: trisomy 7 (the most frequent), loss of chromosome 10, loss of chromosome 22, loss of 9p, 13q; other abnormalities, less frequently described are: gains of chromosomes 1q, 11q, 19, 20, and Xq.

[0055] Glioblastoma multiforme (Grade IV/IV) is the most malignant form of astrocytomas. Although these tumors can occur at almost any age, the peak incidence is between 50 and 70 years old. Glioblastoma multiforme (GBM) is also called a high-grade glioma and is graded by pathologists as Grade IV/IV astrocytoma. These tumors mostly occur in adults with the peak incidence between 50 and 70 years of age. Generally the time from the onset of symptoms to diagnosis is relatively short, usually just a few weeks. Glioblastomas typically show several chromosomal changes: by frequency order, gain of chromosome 7 (50-80% of glioblastomas), double minute chromosomes, total or partial monosomy for chromosome 10 (70% of tumors) associated with the later step in the progression of glioblastomas partial deletion of 9p is frequent (64% of tumors): 9pter-23; partial loss of 22q in 22q13 is frequently reported loss or deletion of chromosome 13, 13q14-q31 is found in some glioblastomas trisomy 19 was reported in glioblastomas by cytogenetic and comparative genomic hybridization (CGH) analysis; the loss of 19q in 19q13.2-qter was detected by loss of heterozygosity (LOH) studies in glioblastomas deletion of chromosome 4q, complete or partial gains of chromosome 20 has been described; gain or amplification of 12q14-q21 has been reported the loss of chromosome Y might be considered, when it occurs in addition to other clonal abnormalities.

[0056] Oligodendrogliomas are benign, slow growing tumors that occur usually in young adults. Often these are located within the frontal lobes which can allow for a safe, complete operative resection. Many oligodendrogliomas contain calcium (little specks of bone) seen best on CT scans.

5 **[0057]** In addition to glioblastomas, the methods of the invention are applicable to a wide variety of other cancers where disregulation of the PI3K/Akt pathway is associated with a concurrent disregulation in cellular growth. For example, in yet another illustrative embodiment of the invention, the disclosed methods and materials are used to examine cancers of the prostate. Utilizing prostate cancer tissue biopsies 10 and/or microarrays, we show that this pathway can be interrogated *in vivo*, using immunohistochemical assays and identify a subset of patients who exhibit complete loss of PTEN protein expression. Loss of PTEN protein expression was significantly correlated with activation of AKT. Activation of AKT was in turn, associated with phosphorylation of S6, in keeping with what is known of the relationships between these 15 signaling molecules. In terms of PTEN biology, it has been shown in genetic systems (i.e. conditional knock-outs) that the dosage of PTEN loss correlates with murine prostate cancer progression. PTEN loss, when examined by Pearson and Multivariate analysis, is significantly associated with phosphorylation of AKT. The phospho-specific antibodies disclosed herein were also used to follow changes in phosphorylation status of these 20 proteins in a prostate cancer cell line, before and after treatment with CCI-779. This highlights the utility of these antibodies to confirm target inhibition in patient samples post treatment.

[0058] As noted above, typical embodiments of the invention examine cellular pathways in the family of tumors termed "prostate cancers". In this context, cancers of 25 the prostate are typically categorized into stages. In *stage T1 (Stage A)*, the tumor is located within the prostate only. It causes no symptoms and is too small to be felt during a digital rectal exam (DRE) or to be seen on an imaging scan. These tumors are usually found by chance during surgery for some other prostate disease, or by following up on screening tests that measure PSA. In *stage T2 (Stage B)*, the tumor is still located 30 within the prostate but is large enough to be felt during a DRE. There are often no

symptoms. In Stage T3 (Stage C), the tumor has spread from the prostate into the immediately surrounding tissue, possibly including the seminal vesicles. In *stage T4, t*he tumor is still within the pelvic region but may have spread farther to areas of the bladder or rectum. In stage *N+ (Stage D1)*, prostate cancer is described as N+ if prostate cancer cells are detected in the lymph nodes in the pelvic area. In *stage M+ (Stage D2)*, a tumor that is M+ has spread beyond the pelvic area to other parts of the body. Bone pain, weight loss, and tiredness are common symptoms.

[0059] Cancers of the prostate are also categorized into "grades" which describe the way the cancer tissue looks when seen under a microscope. The most common system used in the USA to grade the appearance of prostate cancer tissue is called the Gleason grading system. This grading system is based on a number range from 2 to 10. The lower the number, the lower the grade, and the slower the cancer is growing. The higher the score, the higher the grade of the tumor. High-grade tumors grow more quickly than low-grade tumors, and are more likely to spread to other parts of the body. Grades under 4 mean that the cancer cells look similar to your normal cells, and the cancer is likely to be less aggressive. Grades 5 to 7 are in the intermediate range. This means that the cancer cells do not look like normal cells, and are more likely to be aggressive and grow faster. Grades 8 to 10 indicate that the cancer cells are more likely to be very aggressive in growth.

[0060] Certain embodiments of the invention include methods to obtain information used to identify a therapeutic agent for treating a cancer such as a glioblastoma or a prostate cancer in a human. For example, methods of the invention examine the levels of certain polypeptides (e.g. PTEN) and/or the phosphorylation state of certain polypeptides (e.g. S6) to obtain information on how the cancer cell will respond to rapamycin or a rapamycin analog. Rapamycin (also known as sirolimus or rapammune) is a macrolide, related to cyclosporin with immunosuppressive properties and antiproliferative activity in various human tumor cells lines and tumor xenograft models. Both rapamycin and rapamycin analogues with more favorable pharmaceutical properties, such as SDZ-RAD, CCI-779, RAD 001, Everolimus (Certican) and AP23573, are highly specific inhibitors of mTOR. As noted herein the mammalian target of

rapamycin (mTOR) is a downstream effector of the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) signaling pathway that mediates cell survival and proliferation, and consequently is a target for anticancer therapeutic development. In essence, rapamycin and rapamycin analogues gain function by binding to the immunophilin FK506 binding protein 12 and the resultant complex inhibits the activity of mTOR. Because mTOR activates both the 40S ribosomal protein S6 kinase (p70s6k) and the eukaryotic initiation factor 4E-binding protein-1, rapamycin-like compounds block the actions of these downstream signaling elements, which results in cell cycle arrest in the G1 phase. Rapamycin and its analogues also prevent cyclin-dependent kinase (CDK) activation, inhibit retinoblastoma protein phosphorylation, and accelerate the turnover of cyclin D1, leading to a deficiency of active CDK4/cyclin D1 complexes, all of which potentially contribute to the prominent inhibitory effects of rapamycin at the G1/S boundary of the cell cycle. Rapamycin and rapamycin analogues have demonstrated impressive growth-inhibitory effects against a broad range of human cancers. For example, as noted herein, mammalian target of rapamycin (mTOR) modulates key signaling pathways that promote uncontrolled proliferation of glioblastoma multiforme. In this context the methods of the invention can be used to examine the PI3K/Akt pathway and then select an appropriate therapeutic agent in cells having a deregulated PI3K/Akt pathway (e.g. rapamycin). For discussions of Rapamycin and its analogs, see, e.g. Mita et al., Clin Breast Cancer 2003 Jun;4(2):126-37; Hosoi et al., Mol Pharmacol. 1998 Nov;54(5):815-24; Hidalgo et al., Oncogene. 2000 Dec 27;19(56):6680-6; Alexandre et al., Bull Cancer. 1999 Oct;86(10):808-11; and Eshleman et al., Cancer Res. 2002 Dec 15;62(24):7291-7.

[0061] Overexpression of epidermal growth factor receptor (EGFR) is also observed in a wide variety of cancers such as glioma and has frequently been correlated with poor prognosis, thus stimulating efforts to develop new cancer therapies that target EGFR. Monoclonal antibodies and tyrosine kinase inhibitors specifically targeting EGFR are the most well-studied and hold substantial promise of success. Several compounds of monoclonal antibodies and tyrosine kinase inhibitors targeting EGFR have been studied and clinical trials are now underway to test the safety and efficacy of

these targeting strategies in a variety of human cancers. Compounds that target the extracellular ligand-binding region of EGFR include antibodies such as Cetuximab (also known as Erbitux or IMC-C225). Other compounds such as tyrosine kinase inhibitors which target the intracellular domain of EGFR, include ZD-1839 (also known as gefitinib or Iressa), OSI-774 (also known as Erlotinibor or Tarceva), PD-153053, PD-168393 and CI-1033, have been studied in clinical settings alone or in combination with radiation or chemotherapy. In addition, compounds such as h-R3, ABX-EGF, EMD-55900 and ICR-62 have proved to be effective in targeting malignant cells alone or in combination with traditional therapies. The effects of ZD 1839 (Iressa) is currently being studied in clinical trails for patients with glioblastoma multiforme. In this context the methods of the invention can be used to examine the PI3K/Akt pathway and then select an appropriate therapeutic agent in cells that do not have a deregulated PI3K/Akt pathway (e.g. an EGFR inhibitor). For discussions of EGFR inhibitors see, e.g. Khalil et al., Expert Rev Anticancer Ther. 2003 Jun;3(3):367-80; Chakravarti et al., Int J Radiat Oncol Biol Phys. 2003 Oct 1;57(2 Suppl):S329; Wissner et al., Bioorg Med Chem Lett. 2002 Oct 21;12(20):2893-7; Ciardiello et al., Expert Opin Investig Drugs, 2002 Jun;11(6):755-68; De Bono et al., Trends Mol Med. 2002;8(4 Suppl):S19-26; and Cohen, Clin Colorectal Cancer. 2003 Feb;2(4):246-51.

Typical Methods of the Invention

[0062] The invention disclosed herein has a number of embodiments. Illustrative embodiments of the invention include methods which examine tumor samples such as formalin fixed, paraffin embedded glioblastoma multiforme or prostate cancer biopsy samples for evidence of deregulated activation of the PI3K/Akt pathway. These methods involve examining the presence and/or phosphorylation status of the disclosed biomarkers that are associated with this pathway in order to identify and/or assess a therapeutic agent that may be useful in the treatment of such a cancer. As disclosed herein, the presence and/or phosphorylation status of the disclosed biomarkers serves as a marker or proxy of pathway activity.

[0063] Typically, the methods of the invention are used in evaluating the whether a tumor such as a prostate cancer or glioma is likely to respond (i.e. is likely to exhibit growth inhibition) when contacted with an mTOR inhibitor or an EGFR inhibitor. In such embodiments, the presence and/or phosphorylation status of a biomarker polypeptide that is associated with the activation of a pathway (e.g. a phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1)) is examined to determine if the pathway is disregulated in that tumor and is therefore susceptible to inhibition by a inhibitor known to target that pathway. In such embodiments, the tumor is examined prior to its exposure to the inhibitor. Alternatively, the methods evaluate whether a tumor such as a glioma or prostate cancer is responsive (i.e. exhibits growth inhibition) to an mTOR inhibitor or an EGFR inhibitor. In such embodiments, the activity of a biomarker polypeptide that is associated with the activation of a pathway (e.g. a phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1)) is examined after the tumor is exposed to the inhibitor to determine if the biomarkers in the pathway respond to exposure to the inhibitor. As the art that teaches that this growth related pathway is common pathway that is disregulated in a wide variety of human cancers. Consequently, artisans understand that the methods and materials disclosed herein can be universally applied to examine this pathway in all cancers in which the deregulated activation of the PI3K/Akt pathway is observed. In this context, while the use of the disclosed methods and materials in the examination of gliomas and cancers of the prostate represent preferred embodiments of the invention, artisans understand that these are illustrative embodiments and that these methods and materials can be applied to a wide variety of human cancers.

[0064] One such embodiment of the invention is a method for identifying a mammalian glioma (e.g. glioblastoma multiforme) tumor likely to respond, is responsive to an EGFR polypeptide (SEQ ID NO: 7) inhibitor or an mTOR polypeptide (SEQ ID NO: 2) inhibitor, the method comprising examining a sample obtained from the tumor for: the expression of PTEN polypeptide (SEQ ID NO: 5); and the presence of at least one of, a phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1); a EGFR polypeptide (SEQ ID NO: 7); a phosphorylated AKT polypeptide (SEQ ID NO: 4); and

a phosphorylated ERK polypeptide (SEQ ID NO: 8), wherein decreased expression of PTEN polypeptide together with decreased phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the glioma tumor as likely to respond or responsive to an mTOR inhibitor, and wherein decreased expression an of
5 PTEN together with normal phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the glioma tumor as not likely to respond or non-responsive to an mTOR inhibitor, and wherein normal or increased expression of PTEN and increased expression and/or activity of EGFR together with increased phosphorylation of AKT and/or phosphorylation of ERK identifies the glioma tumor as
10 not likely to respond and/or is non-responsive to an EGFR inhibitor. Optionally, the phosphorylation of S6 ribosomal polypeptide is determined subsequent to contacting the tumor or sample with an mTOR inhibitor and/or the phosphorylation of AKT and/or ERK is determined subsequent to contacting the tumor or sample with an EGFR inhibitor. In illustrative embodiments, the mTOR inhibitor is rapamycin, SDZ-RAD,
15 CCI-779, RAD 001, or AP23573 and the EGFR inhibitor is ZD-1839, OSI-774, PD-153053, PD-168393, IMC-C225 or CI-1033.

[0065] Another embodiment of the invention is a method for identifying a mammalian prostate tumor likely to respond, is responsive to an EGFR polypeptide (SEQ ID NO: 7) inhibitor or an mTOR polypeptide (SEQ ID NO: 2) inhibitor, the
20 method comprising examining a sample obtained from the tumor for: the expression of PTEN polypeptide (SEQ ID NO: 5); and the presence of at least one of, a phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1); a EGFR polypeptide (SEQ ID NO: 7); a phosphorylated AKT polypeptide (SEQ ID NO: 4); and a phosphorylated ERK polypeptide (SEQ ID NO: 8), wherein decreased expression of PTEN polypeptide
25 together with decreased phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the prostate tumor as likely to respond or responsive to an mTOR inhibitor, and wherein decreased expression an of PTEN together with normal phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the prostate tumor as not likely to respond or non-responsive to an
30 mTOR inhibitor, and wherein normal or increased expression of PTEN and increased

expression and/or activity of EGFR together with increased phosphorylation of AKT and/or phosphorylation of ERK identifies the prostate tumor as not likely to respond and/or is non-responsive to an EGFR inhibitor. Optionally, the phosphorylation of S6 ribosomal polypeptide is determined subsequent to contacting the tumor or sample with
5 an mTOR inhibitor and/or the phosphorylation of AKT and/or ERK is determined subsequent to contacting the tumor or sample with an EGFR inhibitor. In illustrative embodiments, the mTOR inhibitor is rapamycin, SDZ-RAD, CCI-779, RAD 001, or AP23573 and the EGFR inhibitor is ZD-1839, OSI-774, PD-153053, PD-168393, IMC-C225 or CI-1033.

10 **[0066]** In typical methods, the expression of the biomarker polypeptides is examined using an antibody such as an antibody that binds an epitope comprising a phosphorylated serine residue at position 235 in SEQ ID NO: 1, an antibody that binds an epitope comprising a phosphorylated serine residue at position 473 in SEQ ID NO: 4, or an antibody that binds an epitope comprising a phosphorylated threonine residue at
15 position 202 and tyrosine 204 in SEQ ID NO: 8. Optionally, the sample is a paraffin embedded biopsy sample.

[0067] Another embodiment of the invention is a method for identifying a mammalian tumor that does not express a PTEN polypeptide (SEQ ID NO: 5) and which is not likely to respond or is nonresponsive to an inhibitor of mTOR polypeptide
20 (SEQ ID NO: 2) activity, the method comprising examining a sample obtained from the tumor for the presence of phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1) after contacting the tumor or the sample with the inhibitor, wherein, an observable decrease in phosphorylated S6 ribosomal polypeptide in the sample, as compared to a control that is not contacted with the inhibitor identifies the tumor as likely to respond
25 or responsive to the inhibitor, and wherein no observable decrease in phosphorylated S6 ribosomal polypeptide in the sample, as compared to a control identifies the tumor as not likely to respond or nonresponsive to the inhibitor.

[0068] Yet another embodiment of the invention is a method for identifying a mammalian tumor that expresses a PTEN polypeptide (SEQ ID NO: 5) and which is not
30 likely to respond or is nonresponsive to an inhibitor of EGFR polypeptide (SEQ ID

NO: 7) activity, the method comprising examining a sample obtained from the tumor for the presence of EGFR (SEQ ID NO: 7) and the presence of a phosphorylated AKT polypeptide (SEQ ID NO: 4) or the presence of a phosphorylated ERK polypeptide (SEQ ID NO: 8) after contacting the tumor or the sample with the inhibitor, wherein an increase in the levels of the EGFR polypeptide and the levels of phosphorylated AKT polypeptide or phosphorylated ERK polypeptide identifies the tumor as not likely to respond or nonresponsive to the inhibitor. Optionally, the sample obtained from the tumor is examined for the presence of a phosphorylated AKT polypeptide (SEQ ID NO: 4) and the presence of a phosphorylated ERK polypeptide (SEQ ID NO: 8).

[0069] As noted above, certain embodiments of the invention include the examination of the expression of a polypeptide or phosphorylation of a polypeptide. As is known in the art, the examination of such polypeptide expression and polypeptide phosphorylation status in a cell or tissue sample is typically evaluated as compared to a control, i.e. a control cell and/or tissue sample that has a defined or predetermined level of polypeptide expression or phosphorylation. In an example of polypeptide phosphorylation, a control can be a normal tissue (e.g. non cancerous glial cells) where it is observed that a polypeptide is typically not phosphorylated. In an example of polypeptide expression, Example 3 and Figure 2 provided illustrative examples of the methods of the invention using such controls, in particular, a PTEN expression grading system known the art that uses vascular endothelium as a control. Specifically PTEN immunohistochemical staining (which is directly correlated with PTEN expression) is scored according to an established scale of 0-2, in which the vascular endothelium (score of 2) serves as an internal control. Tumor cells are graded as 2 if their staining intensity is equal to that of the vascular endothelium, 1 if it is diminished relative to the vascular endothelium, and 0 if it is undetectable in the tumor cells and present in the vascular endothelium. This scoring system, which has been shown to be highly consistent between different cancer cell types, including gliomas (as disclosed herein) and cancers of the breast, ovary, pancreas and colon, allows artisans to readily examine the expression levels of PTEN polypeptides in a sample such as a formalin fixed, paraffin embedded biopsy sample.

[0070] Additional embodiments of the invention include a method for identifying a mammalian cancer cell that does not express a PTEN polypeptide (SEQ ID NO: 5) and which is likely to exhibit growth inhibition when contacted with an inhibitor of mTOR polypeptide (SEQ ID NO: 2) activity, the method comprising examining the cancer cell for the presence of phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1) after contacting the cancer cell with the inhibitor, wherein, an observable decrease in phosphorylated S6 ribosomal polypeptide in the sample, as compared to a control mammalian cancer cell that is not contacted with the inhibitor identifies the cancer cell as likely to exhibit growth inhibition when contacted with the inhibitor, and further wherein no observable decrease in phosphorylated S6 ribosomal polypeptide in the sample, as compared to a control mammalian cell identifies the cancer cell as not likely to exhibit growth inhibition when contacted with the inhibitor. In these methods, the inhibitor of mTOR polypeptide activity is optionally rapamycin, CCI-779, RAD 001, or AP23573. Typically, the expression of the PTEN polypeptide or the presence of phosphorylated S6 ribosomal polypeptide is examined using an antibody that binds the PTEN polypeptide or the phosphorylated S6 ribosomal polypeptide (e.g. an antibody that binds an epitope comprising a phosphorylated serine residue at position 235 in SEQ ID NO: 1). Preferably, the mammalian cancer cell is obtained from a paraffin embedded biopsy sample.

[0071] Another embodiment of the invention is a method for identifying a mammalian cancer cell that expresses a PTEN polypeptide (SEQ ID NO: 5) and which is not likely to exhibit growth inhibition when contacted with inhibitor of EGFR polypeptide (SEQ ID NO: 7) activity, the method comprising examining the cancer cell for the presence of EGFR (SEQ ID NO: 7), the presence of a phosphorylated AKT polypeptide (SEQ ID NO: 4) or a the presence of a phosphorylated ERK polypeptide (SEQ ID NO: 8), wherein an increase in the levels of the EGFR polypeptide and the levels of phosphorylated AKT polypeptide or phosphorylated ERK polypeptide identifies the cancer cell as not likely to exhibit growth inhibition when contacted with inhibitor of the EGFR polypeptide. In these methods, the inhibitor of EGFR activity is optionally ZD-1839, OSI-774, PD-153053, PD-168393 or CI-1033. Typically, the

expression of the PTEN polypeptide or the presence of EGFR polypeptide is examined using an antibody that binds the PTEN polypeptide or the EGFR polypeptide. Optionally, the presence of phosphorylated AKT is examined using an antibody that binds an epitope comprising a phosphorylated serine residue at position 473 in SEQ ID NO: 4 and the presence of phosphorylated ERK is examined using an antibody that binds an epitope comprising a phosphorylated threonine residue at position 202 or a phosphorylated tyrosine residue at position 204 in SEQ ID NO: 8. In illustrative methods, the mammalian cancer cell is obtained from a paraffin embedded biopsy sample.

[0072] Another embodiment of the invention is a method for determining the responsiveness of a mammalian cancer cell to a growth inhibitory agent selected from the group consisting of a EGFR polypeptide (SEQ ID NO: 7) inhibitor or an mTOR polypeptide (SEQ ID NO: 2) inhibitor, the method comprising examining the glioblastoma cell for the presence of a S6 polypeptide (SEQ ID NO: 1) having a phosphorylated serine, threonine or tyrosine residue; a mTOR polypeptide (SEQ ID NO: 2) having a phosphorylated serine, threonine or tyrosine residue; a FKHR polypeptide (SEQ ID NO: 3) having a phosphorylated serine, threonine or tyrosine residue; a AKT polypeptide (SEQ ID NO: 4) having a phosphorylated serine, threonine or tyrosine residue; a ERK polypeptide (SEQ ID NO: 8) having a phosphorylated serine, threonine or tyrosine residue; or the expression of the PTEN polypeptide (SEQ ID NO: 5), wherein the presence of a phosphorylated S6, mTOR, FKHR, AKT or ERK polypeptide, or decreased levels of expression of the PTEN polypeptide in the glioblastoma cell relative to a control mammalian vascular endothelial cell determines the responsiveness of the mammalian cancer cell to the growth inhibitory agent. Optionally in such methods, the mammalian cancer cell has been contacted with the growth inhibitory agent. Alternatively, the mammalian cancer cell has not been contacted with the growth inhibitory agent.

[0073] Yet another embodiment of the invention is a method to obtain information used to identify a therapeutic agent for treating glioblastoma in a human, the method comprising examining a cancer cell obtained from the human for the presence

of: a S6 polypeptide (SEQ ID NO: 1) having a phosphorylated serine, threonine or tyrosine residue; a mTOR polypeptide (SEQ ID NO: 2) having a phosphorylated serine, threonine or tyrosine residue; a FKHR polypeptide (SEQ ID NO: 3) having a phosphorylated serine, threonine or tyrosine residue; a AKT polypeptide (SEQ ID NO: 4) having a phosphorylated serine, threonine or tyrosine residue; or decreased levels of expression of the PTEN polypeptide (SEQ ID NO: 5), wherein the presence of a phosphorylated S6, mTOR, FKHR or AKT polypeptide, or decreased levels of expression of the PTEN polypeptide in the cancer cell provides information used to identify a therapeutic agent for treating the cancer in the human. Optionally in this method, the cancer cell is examined for the presence of a plurality of these characteristics. In one such embodiment, the cancer cell is examined for the presence of a S6 polypeptide (SEQ ID NO: 1) having a phosphorylated serine, threonine or tyrosine residue and decreased levels of expression of the PTEN polypeptide (SEQ ID NO: 5). Optionally the cancer cell is in a paraffin embedded biopsy sample.

[0074] As noted above, embodiments of the invention typically utilize antibodies that specifically bind phosphorylated polypeptides, i.e. polypeptides having a phosphorylated serine, threonine or tyrosine residue. In this context the disclosure provides antibodies that bind to specific epitopes comprising a phosphorylated residue (e.g. serine at position 2481 in SEQ ID NO: 2). By utilizing antibodies that bind to an epitope that comprises a phosphorylated residue (i.e. phospho-specific antibodies) but which do not bind to the unphosphorylated form of the same polypeptide, these phospho-specific antibodies can be used to examine the activation status of a pathway, where the activation is associated with phosphorylation of one or more specified residues. In certain embodiments of the invention, the phosphorylation status and/or expression levels of multiple members of a signalling pathway (e.g. S6 and mTOR) are examined as a confirmatory assessment of the signalling cascade associated with the pathway.

[0075] Certain embodiments of the invention are used with formalin fixed, paraffin embedded biopsy samples. In particular, the disclosure provided herein demonstrates that antibodies such as phospho-specific antibodies can be used with

antigen samples processed in this manner. Significantly, the disclosure provided herein further demonstrates that the methods using these samples provide an accurate demonstration of the physiological status of the pathways in these samples. Consequently, the disclosure provided herein demonstrates how the methods of the invention are well suited for use with commonly available clinical samples.

[0076] In one illustrative embodiment of the invention, the presence of a S6 polypeptide (SEQ ID NO: 1) having a phosphorylated serine, threonine or tyrosine residue is examined using an antibody that binds an epitope comprising a phosphorylated serine residue at position 235 in SEQ ID NO: 1. In another illustrative embodiment of the invention, the presence of a mTOR polypeptide (SEQ ID NO: 2) having a phosphorylated serine, threonine or tyrosine residue is examined using an antibody that binds an epitope comprising a phosphorylated serine residue at position 2481 in SEQ ID NO: 2. In another illustrative embodiment of the invention, the presence of a FKHR polypeptide (SEQ ID NO: 3) having a phosphorylated serine, threonine or tyrosine residue is examined using an antibody that binds an epitope comprising a phosphorylated threonine residue at position 24 in SEQ ID NO: 3. In another illustrative embodiment of the invention, the presence of a AKT polypeptide (SEQ ID NO: 4) having a phosphorylated serine, threonine or tyrosine residue is examined using an antibody that binds an epitope comprising a phosphorylated serine residue at position 473 in SEQ ID NO: 4. The expression levels and/or phosphorylation of additional polypeptide markers can also be examined. Illustrative example of such additional markers include Ki-67 (SEQ ID NO: 9) and p-H3 histone H3 (SEQ ID NO: 10).

[0077] Yet another embodiment of the invention is a method of examining a mammalian cell for evidence of Akt pathway activation comprising examining the mammalian cell for the presence of: a S6 polypeptide (SEQ ID NO: 1) having a phosphorylated serine residue at position 235 in SEQ ID NO: 1; a mTOR polypeptide (SEQ ID NO: 2) having a phosphorylated serine residue at position 2481 in SEQ ID NO: 2; a FKHR polypeptide (SEQ ID NO: 3) having a phosphorylated threonine residue at position 24 in SEQ ID NO: 3; a AKT polypeptide (SEQ ID NO: 4) having a phosphorylated serine residue at position 473 in SEQ ID NO: 4; or decreased levels of

expression of the PTEN polypeptide (SEQ ID NO: 5), wherein the presence of a phosphorylated S6, mTOR, FKHR or AKT polypeptide, or decreased levels of expression of the PTEN polypeptide evidence of Akt pathway activation in the mammalian cell. Optionally the mammalian cell is examined for the presence of a plurality of characteristics such as a S6 polypeptide (SEQ ID NO: 1) having a phosphorylated serine residue at position 235 in SEQ ID NO: 1 and decreased levels of expression of the PTEN polypeptide (SEQ ID NO: 5). Typically in this method, the mammalian cell is a cancer cell such as a cancer cell is of a glioblastoma lineage.

[0078] Certain embodiments of the invention comprise further methodological steps, for example using the results of the examination in a prognostic determination of tumor progression and/or using the results of the examination to identify the presence of a glioblastoma characterized by a short time from initial diagnosis to patient death. Optionally the further methodological steps include the step of using the results of the examination to identify a therapeutic agent for treating the glioblastoma such as the step of using the results of the examination to evaluate the effect of rapamycin on the glioblastoma cancer cell. Optionally the mammalian cell is in a paraffin embedded biopsy sample.

[0079] A preferred embodiment of the invention is a method of examining a mammalian cell for evidence of Akt pathway activation comprising using a phospho-specific antibody to examine the cell for the presence of a phosphorylated protein in the mammalian cell selected from the group consisting of mTOR, FKHR and S6, wherein the presence of a phosphorylated mTOR, FKHR or S6 protein in the mammalian cell provides evidence of Akt pathway activation. In highly preferred embodiments, the cell is examined for the concurrent phosphorylation of mTOR, FKHR S6 proteins. Such methods typically include an optional step of using a phospho-specific antibody to examine the cell for evidence of phosphorylation of a Akt protein in the mammalian cell. In such methods, the mammalian cell is typically a cancer cell that is present in a paraffin embedded biopsy sample. In highly preferred embodiments of the invention the cancer cell is of the glioblastoma lineage.

[0080] Yet another embodiment of the invention is a method of examining a mammalian cell for evidence of Erk pathway activation comprising using a phospho-specific antibody to examine the cell for presence of phosphorylated p-44/42 MAP kinase proteins in the cells, wherein the presence of phosphorylated p-44/42 MAP kinase proteins in the mammalian cell provides evidence of Erk pathway activation. In preferred methods, the mammalian cell is present in a paraffin embedded biopsy sample obtained from an individual suspected of suffering from glioblastoma.

[0081] Another embodiment of the invention is a method of examining a tissue sample for the presence of mammalian glioblastoma cells having a phenotype characterized by a shorter time to tumor progression comprising using phospho-specific antibodies to examine the cell for the presence of phosphorylated mTOR, FKHR and S6 proteins in the cells, wherein the presence of a phosphorylated mTOR, FKHR and S6 proteins in the mammalian cell provides evidence of the phenotype. A related embodiment of the invention is a method of examining a tissue sample for the presence of mammalian glioblastoma cells having a phenotype characterized by a short time from initial diagnosis to patient death comprising using phospho-specific antibodies to examine the cell for the presence of phosphorylated mTOR, FKHR and S6 proteins in the cells, wherein the presence of a phosphorylated mTOR, FKHR and S6 proteins in the mammalian cell provides evidence of the phenotype.

[0082] Another embodiment of the invention is a method of examining a tissue sample for the presence of mammalian glioblastoma cells having a phenotype characterized by a shorter time to tumor progression comprising using a phospho-specific antibody to examine the cell for the presence of phosphorylated Erk proteins in the cells, wherein the presence of a phosphorylated Erk proteins in the mammalian cell provides evidence of the phenotype. A related embodiment of the invention is a method of examining a tissue sample for the presence of mammalian glioblastoma cells having a phenotype characterized by a short time from initial diagnosis to patient death comprising using phospho-specific antibodies to examine the cell for the presence of phosphorylated p-44/42 MAP kinase proteins in the cells, wherein the presence of a

phosphorylated p-44/42 MAP kinase proteins in the mammalian cell provides evidence of the phenotype.

[0083] Yet another embodiment of the invention is a method of obtaining information useful for identifying an appropriate therapeutic agent to use to treat an individual suffering from glioblastoma comprising examining a tissue sample from the patient for the presence of glioblastoma cells having a phosphorylated protein selected from the group consisting of mTOR, FKHR and S6, wherein the presence of a phosphorylated mTOR, FKHR or S6 protein in the mammalian cell provides information useful for identifying an appropriate therapeutic agent to use to treat an individual suffering from glioblastoma. In preferred embodiments of the invention the mammalian cell is examined for the presence of at least two and more preferably three phosphorylated proteins selected from the group consisting of mTOR, FKHR and S6. Typically the therapeutic agent is a kinase inhibitor of the Akt pathway.

[0084] Another embodiment of the invention is a method of obtaining information useful for identifying an appropriate therapeutic agent to use to treat an individual suffering from glioblastoma comprising examining a tissue sample from the patient for the presence of glioblastoma cells having phosphorylated Erk proteins, wherein the presence of phosphorylated Erk proteins in the mammalian cell provides information that can be used to identify an appropriate therapeutic agent to use to treat an individual suffering from glioblastoma.

[0085] Another embodiment of the invention is a method of examining a mammalian cell for evidence of Akt pathway activation comprising examining the cell for the expression of the EGFR and the EGFRvIII proteins, wherein the coexpression of the EGFR and the EGFRvIII proteins in the cell provides evidence of Akt pathway activation. A related embodiment of the invention is a method of examining a mammalian cell for evidence of Erk pathway activation comprising examining the cell for the expression of the EGFR and the EGFRvIII proteins, wherein the coexpression of the EGFR and the EGFRvIII proteins in the cell provides evidence of Erk pathway activation. Yet another embodiment of the invention is a method of examining a mammalian glioblastoma cell for evidence of Akt pathway activation, wherein the

mammalian glioblastoma cell is obtained from a paraffin embedded biopsy sample, the method comprising examining the cell for decreased expression of the PTEN protein, wherein a decrease in the expression of the PTEN protein cell provides evidence of Akt pathway activation.

5

Articles of Manufacture of the Invention

[0086] Embodiments of the invention also include articles of manufacture and/or kits designed to facilitate the methods of the invention. Typically such kits include instructions for using the elements therein according to the methods of the
10 present invention. Such kits can comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. For example, one of the container means can comprise one or more of the antibodies disclosed herein (an anti-S6 antibody for example) that is or can be detectably
15 labeled with a marker. For kits utilizes immunological methods (e.g. immunohistochemistry and Western blotting) to detect the target proteins, the kit can also have containers containing buffers for these methods and/or containers comprising antibodies labelled with a reporter-means, such as a chromophore or radioactive molecule. In addition, for kits which utilize additional methodologies such as caspase-3
20 assays or tunel assays of apoptosis, additional reagents associated with these techniques can be further included in the kits.

[0087] In a typical embodiment of the invention, an article of manufacture containing materials useful for the examination of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable
25 containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container can hold a composition (e.g. an antibody composition) which is effective for examining mammalian cells (e.g. prostate cancer or glioblastoma cells). The label on, or associated with, the container indicates that the composition is used for examining cellular
30 polypeptides. The article of manufacture may further comprise a second container

comprising a buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

5 **[0088]** One such embodiment of the invention is a kit comprising at least one antibody selected from the group consisting of: an antibody that binds a S6 polypeptide (SEQ ID NO: 1), wherein the S6 polypeptide epitope bound by the antibody comprises a phosphorylated serine, threonine or tyrosine residue; an antibody that binds a mTOR polypeptide (SEQ ID NO: 2), wherein the mTOR polypeptide epitope bound by the 10 antibody comprises a phosphorylated serine, threonine or tyrosine residue; an antibody that binds a FKHR polypeptide (SEQ ID NO: 3), wherein the FKHR polypeptide epitope bound by the antibody comprises a phosphorylated serine, threonine or tyrosine residue; and an antibody that binds a AKT polypeptide (SEQ ID NO: 4), wherein the AKT polypeptide epitope bound by the antibody comprises a phosphorylated serine, 15 threonine or tyrosine residue; and wherein the kit further includes instructions for using the antibody to examining a mammalian cell for evidence of AKT pathway activation. Optionally the kit further comprises an antibody that binds a PTEN polypeptide (SEQ ID NO: 5). The kits of the invention can further include antibodies to additional polypeptides such as Ki-67 (SEQ ID NO: 9) and p-H3 histone H3 (SEQ ID NO: 10).

20 **[0089]** Another embodiment of the invention is a kit comprising an antibody capable of immunospecifically binding a phosphorylated protein in a mammalian cell selected from the group consisting of phosphorylated Akt, mTOR, FKHR and S6 proteins and instructions for using the antibody to examining the mammalian cell for evidence of Akt pathway activation. In preferred methods, the kit comprises different 25 antibodies, each of which is capable of immunospecifically binding 2, 3 or 4 phosphorylated proteins in a mammalian cell selected from the group consisting of phosphorylated Akt, mTOR, FKHR and S6 proteins. Another embodiment of the invention is a kit comprising an antibody capable of immunospecifically binding a phosphorylated p-44/42 MAP kinase proteins in a mammalian glioblastoma cell present

in a paraffin embedded biopsy sample and instructions for using the antibody to examining the mammalian cell for evidence of Erk pathway activation.

[0090] Yet another embodiment of the invention is a kit for characterizing a mammalian prostate cancer or glioblastoma (GBM) tumor or cell, the kit comprising: an antibody that binds PTEN (SEQ ID NO: 5) and at least on of the following: an antibody that binds phosphorylated S6 ribosomal protein (SEQ ID NO: 1); an antibody that binds EFGR (SEQ ID NO: 7); an antibody that binds phosphorylated AKT (SEQ ID NO: 4); and/or an antibody that binds phosphorylated ERK (SEQ ID NO: 8); and at least one secondary antibody that binds to the above noted primary antibodies. Optionally the kit comprises a plurality of these antibodies. In a specific embodiment, the kit includes an antibody specific for S6 ribosomal protein (SEQ ID NO: 1) having a phosphorylated serine residue at position 235 in SEQ ID NO: 1; an antibody specific for AKT (SEQ ID NO: 4) having a phosphorylated serine residue at position 473 in SEQ ID NO: 4; or an antibody specific for ERK (SEQ ID NO: 8) having a phosphorylated threonine residue at position 202 or a phosphorylated tyrosine residue at position 204 in SEQ ID NO: 8.

[0091] Another embodiment of the invention is a kit for characterizing a mammalian prostate cancer or glioma tumor or cell, the kit comprising: an antibody that binds PTEN (SEQ ID NO: 5); an antibody that binds phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1); an antibody that binds EFGR (SEQ ID NO: 7); an antibody that binds phosphorylated AKT (SEQ ID NO: 4); an antibody that binds phosphorylated ERK (SEQ ID NO: 8). Typically the kit further comprises a secondary antibody which binds to one of the primary antibodies directed to these polypeptides. Optionally the kit comprises a plurality of antibodies such as an antibody specific for S6 ribosomal polypeptide (SEQ ID NO: 1) having a phosphorylated serine residue at position 235 in SEQ ID NO: 1, an antibody specific for AKT (SEQ ID NO: 4) having a phosphorylated serine residue at position 473 in SEQ ID NO: 4; or antibody specific for ERK having a phosphorylated threonine residue at position 202 and tyrosine 204 in SEQ ID NO: 8. Optionally the kit further includes an antibody that binds Ki-67 polypeptide (SEQ ID NO: 9), p-H3 histone polypeptide (SEQ ID NO: 10) or caspase-3 polypeptide (SEQ ID NO: 11).

Typical Protocols Useful To The Practice Of The Invention

[0092] The methods of the present invention typically utilize antibodies directed to polypeptides in the PI3K/Akt pathway. Illustrative antibody compositions useful in the present invention are anti-phosphoprotein antibodies characterized as containing antibody molecules that specifically immunoreacts with a phosphorylated form of a polypeptide associated with the PI3K/Akt pathway. The polypeptide may be for example, S6, mTOR, FKHR, AKT or PTEN. By "specifically immunoreacts", it is meant that the antibody binds to the phosphorylated form of polypeptide (i.e. is phospho-specific) and does not bind to the unphosphorylated form of the same polypeptide. Consequently, the phosphorylation associated with pathway activation can be examined with such antibodies. Therefore, the antibodies of the invention can distinguish between the phosphorylated and unphosphorylated forms of a polypeptides associated with the PI3K/Akt pathway. Consequently, the phosphorylation associated with pathway activation can be examined with such antibodies. Typically the assays of the invention include immunohistochemical techniques using the antibodies disclosed herein. For example, a sample can be examined for the presence of a biochemical pathway associated phosphorylated polypeptide such as phosphorylated ERK by using an antibody that binds an epitope comprising a phosphorylated threonine residue at position 202 and tyrosine 204 in SEQ ID NO: 8.

1. Antibodies

[0093] The antibodies useful in the invention may comprise polyclonal antibodies, for example affinity purified polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the appropriate polypeptide epitopes (e.g. a S6 polypeptide (SEQ ID NO: 1) having a phosphorylated serine, threonine or tyrosine residue, a mTOR

polypeptide (SEQ ID NO: 2) having a phosphorylated serine, threonine or tyrosine residue, a FKHR polypeptide (SEQ ID NO: 3) having a phosphorylated serine, threonine or tyrosine residue, a ERK polypeptide (SEQ ID NO: 8) having a phosphorylated serine, threonine or tyrosine residue, a AKT polypeptide (SEQ ID NO: 5 4) having a phosphorylated serine, threonine or tyrosine residue, or a PTEN polypeptide) or a fusion protein thereof.

[0094] In addition, it may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum 10 albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

[0095] The antibodies may, alternatively, be monoclonal antibodies. 15 Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, <u>Nature</u>, <u>256</u>:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the 20 lymphocytes may be immunized *in vitro*.

[0096] The immunizing agent will typically include a phosphorylated S6, mTOR, FKHR, ERK or AKT polypeptide or PTEN polypeptide or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian 25 sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, <u>Monoclonal Antibodies: Principles and Practice</u>, Academic Press, (1986) pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are 30 employed. The hybridoma cells may be cultured in a suitable culture medium that

preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and 5 thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

[0097] Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are 10 murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques 15 and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63).

[0098] The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against phosphorylated S6, mTOR, FKHR, ERK or AKT polypeptides or PTEN and EGFR polypeptides. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma 20 cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

25 **[0099]** After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, supra). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal. The monoclonal antibodies secreted by the 30 subclones may be isolated or purified from the culture medium or ascites fluid by

conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0100] The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison et al., supra) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

[0101] The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

[0102] *In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

[0103] Reactivity of antibodies with the cognate protein can be established by a number of well known means, including Western blot, immunoprecipitation, ELISA, and FACS analyses. A antibody or fragment thereof can be labeled with a detectable marker or conjugated to a second molecule. Suitable detectable markers include, but are not limited to, a radioisotope, a fluorescent compound, a bioluminescent compound, chemiluminescent compound, a metal chelator or an enzyme.

2. Assays

[0104] The invention provides assays for examining cellular pathways associated with disregulated cell growth. Certain embodiments of the invention include the steps of detecting the presence of phosphorylated S6, mTOR, FKHR, AKT or ERK polypeptides or PTEN and EGFR polypeptides in a tissue. Methods for detecting these polypeptides are well known and include, for example, immunoprecipitation, immunohistochemical analysis, Western blot analysis, molecular binding assays, ELISA, ELIFA and the like.

[0105] Typically the assays of the invention include immunohistochemical techniques. Immunohistochemical techniques as used herein encompasses the use of reagents detecting cell specific markers, such reagents include, for example antibodies. Antibodies, including monoclonal antibodies, polyclonal antibodies and fragments thereof, are often used to identify proteins or polypeptides of interest in a sample. A number of techniques are utilized to label objects of interest according to immunohistochemical techniques. Such techniques are discussed in Current Protocols in Molecular Biology, Unit 14 et seq., eds. Ausubel, et al., John Wiley & Sons, 1995, the disclosure of which is incorporated herein by reference. Typical protocols include staining a paraffin embedded tissue section prepared according to a conventional procedure (see, e.g. U.S. Patent No. 6,631,203).

[0106] Certain embodiments of the invention include tunel assays a markers of apoptosis. Typically, a *TUNEL* assay is performed essentially as follows: the percentage of apoptotic cells are detected by the APO-BRDU terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling assay (see, e.g. Gavrieli, et al., J. Cell Biol. 119: 493-501) according to manufacturer's instructions (see, e.g. Phoenix Flow Systems,

Phoenix, AZ). For further discussions of TUNEL assays useful in methods of the invention see, e.g. Prochazkova et al., Biotechniques 2003 Sep;35(3):528-34; Duan et al., J Pathol. 2003 Feb;199(2):221-8; and Walker et al., J Pathol. 2001 Oct;195(3):275-6.

[0107] Certain embodiments of the invention include caspase-3 assays. Those skilled in the art will appreciate that the caspase-3 assay measures the activation of caspase-3 enzyme, a critical early event of apoptosis induced death (see, e.g. U.S. Patent Application No. 20020159996 and U.S. Patent No. 6,346,607). For further discussions of TUNEL assays useful in methods of the invention see, e.g. Duan et al., J Pathol. 2003 Feb;199(2):221-8; and Walker et al., J Pathol. 2001 Oct;195(3):275-6.

[0108] Throughout this application, various publications are referenced. The disclosures of these publications are hereby incorporated by reference herein in their entireties.

EXAMPLES

[0109] Examples 1-8 provide illustrative disclosure showing embodiments of the invention used to examine glioma cells. Example 9 provides an illustrative disclosure showing embodiments of the invention used to examine prostate cancer cells.

EXAMPLE 1: Patient selection and construction of Tissue Microarray:

[0110] All patients participating in this study gave informed consent prior to surgery, in accordance with UCLA Institutional Review Board Policies. Formalin-fixed, paraffin-embedded tissue blocks were taken from 45 patients diagnosed with a glioblastoma at initial surgical resection and treated by the UCLA neuro-oncologist. The diagnosis was confirmed independently by at least two Neuropathologists. None of the patients were treated prior to removal of the tumor. Three representative 0.6 mm cores were obtained from diagnostic areas of the tumor blocks from each of the primary GBM patients; two from geographically distinct regions of tumor and one from a region of normal brain tissue when available (approximately 2/3 of cases). The cores were then inserted into a grid pattern in a recipient paraffin block using a tissue arrayer. Five-micron sections were cut from the tissue array and immunohistochemistry was performed. Serial sections from the tissue array were used for immunohistochemical

analysis. Four tumors had sufficient material on the tissue array for analysis of PTEN, EGFR and EGFRvIII, but lacked sufficient material for analysis of p-Akt, p-mTOR, p-S6, p-FKHR and p-Erk.

5 **EXAMPLE 2: Immunohistochemical Staining**

[0111] Sections from the tissue microarray were stained with monoclonal antibodies to PTEN (clone 6H2.1, Cascade Bioscience, Winchester MA), EGFR (clone 31G7, Zymed, San Francisco, CA), EGFRvIII (clone L8A4, a generous gift from Dr. Darrell Bigner), and phosphorylation specific antibodies directed against p-Akt (ser 473)
10 p-FKHR (thr24) /p-FKHRL1 (thr32), p-mTOR (ser 2481), p-S6 ribosomal protein (ser 235/236) and p-44/42 MAP kinase (p-Erk) (thr202/tyr204) (Cell Signaling Technologies, Beverly, MA). Sections were baked at 60°C and de-paraffinized with xylenes and graded ethanols. Heat-induced antigen retrieval was used as follows: for p-Erk, p-Akt, p-mTOR, p-FKHR/FKHRL1 and p-s6, 0.01 M citrate buffer, pH 6 for 25 minutes in a
15 pressure cooker; for PTEN, 0.01M citrate buffer, pH 6 for 16 minutes in a microwave oven; EGFR, pronase (0.03 g/ml 0f 0.05 M Tris buffer, pH 7.4) at 37°C for 8 minutes and for EGFRvIII, 0.01 M citrate buffer, pH 6 for 25 minutes in a vegetable steamer. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. Primary antibodies (PTEN at 1:400, EGFR at 1:150, EGFRvIII at 1:400, p-Akt 1:50, p-
20 mTOR 1:50; p-FKHR/FKHRL1 1:50, pS6 1:50 and p-ERK at 1:50) were diluted in Tris buffered saline with 0.1% Tween and applied for 16 hours at 4°C, followed by anti-mouse or anti-rabbit biotinylated immunoglobulins (Vector) at 1:100 dilution for one hour, and finally, avidin-biotin complex (Elite ABC, Vector) for one hour. Negative control slides received normal mouse serum (DAKO) as the primary antibody.
25 Diaminobenzidine tetrahydrochloride was used as the enzyme substrate to visualize specific antibody localization for PTEN, EGFR and EGFRvIII; Vector NovaRed (Vector) was used for phospho-specific antibodies. Slides were counterstained with Harris hematoxylin.

EXAMPLE 3: Scoring and Interpretation of Immunohistochemistry:

[0112] *PTEN* - PTEN staining was scored according to a previously established scale of 0-2, in which the vascular endothelium (score of 2) serves as an internal control (see, e.g., Perren et al., Am J Pathol. *157:* 1097-103., 2000; Perren et al., Am J Pathol. *155:* 1253-60., 1999; Zhou et al., Am J Pathol. *161:* 439-47., 2002; Gimm et al., Am J Pathol. *156:* 1693-700., 2000). Tumor cells are graded as 2 if their staining intensity is equal to that of the vascular endothelium, 1 if it is diminished relative to the endothelium, and 0 if it is undetectable in the tumor cells and present in the vascular endothelium (see, e.g., Zhou et al., Am J Pathol. *161:* 439-47., 2002). This scoring system has been shown to be highly consistent between different cancer cell types, including breast (see, e.g., Perren et al., Am J Pathol. *155:* 1253-60., 1999), ovarian (see, e.g., Mutter et al., Cancer Res. *61:* 4311-4314., 2001), pancreas (see, e.g., Perren et al., Am J Pathol. *157:* 1097-103., 2000) and colon (see, e.g., Zhou et al., Am J Pathol. *161:* 439-47., 2002). Two Neuropathologists scored the tumors independently. In addition, tumors were scored by one of the Neuropathologists on two independent occasions. Both the inter-rater, and the intra-rater agreement were greater than 90%.

[0113] *EGFR and EGFRvIII* – Tumors demonstrating strong EGFR immunopositivity in greater than 20% of tumor cells were considered to be positive (see, e.g., Liotta et al., Jama. *286:* 2211-4., 2001); tumors demonstrating at least focal moderate to strong immunoreactivity for EGFRvIII were considered positive, as previously reported (see, e.g., Choe et al., Clin Cancer Res. *8:* 2894-901., 2002). The inter-rater and intra-rater agreement for EGFR and EGFRvIII were > 90%.

[0114] *Phosphorylation specific antibodies-* Phospho-Akt, mTOR, S6 and FKHR were scored on a scale of 0-2 (0+ no staining, 1+ = mild intensity cytoplasmic staining, and 2+ = strong cytoplasmic staining; staining of 1+ and 2+ were considered positive. The agreement between reviewers, as well as for the same reviewer on independent reviews, was 80% for p-Akt. It was higher for phosphorylated mTOR, S6 and FKHR, ranging from 87% for mTOR to 100% for S6. For phospho-ERK, tumors that focally contained greater than 5% positive nuclear staining were considered positive, as previously reported

(see, e.g., Choe et al., Clin Cancer Res. *8:* 2894-901., 2002). The agreement between reviewers, and for the same reviewer on independent reviews, was > 85%.

EXAMPLE 4: Statistical analysis

[0115] The association between markers was analyzed using Fisher's Exact test.
5 The software was available on the Simple Interactive Statistical Website which can be identified with a internet search using the terms "home.clara.net" (http://home.clara.net/sisa/index.htm). For analysis of prognostic factors, we excluded 13 patients who did not receive therapy other than surgery. These patients had a poor performance status at the time of diagnosis and elected not to have further therapy. All
10 other patients had received at least standard involved field fractionated radiation therapy. Kaplan-Meier curves were generated to assess the association of variables with time from initial diagnosis to evidence of progression by imaging or clinical features (time to tumor progression) and time from initial diagnosis to death (overall survival). To identify statistically significant differences in time to progression and overall survival, the
15 Wilcoxon two sample test was used.

EXAMPLE 5: Assessment of PTEN/Akt pathway by IHC

[0116] We constructed a tissue microarray consisting of samples from 45 untreated primary GBM patients (Table 1). All of the tumors presented as *de novo* grade IV tumors ("primary GBMs") (see, e.g., Kleihues et al., Neuro-oncol. *1:* 44-51., 1999).
20 None of the patients received any radiation or chemotherapy prior to surgical resection. We focused on primary GBMs because they have a high incidence of PTEN mutations and EGFR over-expression (see, e.g., Kleihues et al., Neuro-oncol. *1:* 44-51., 1999) and because this enabled us to analyze PI3'K/Akt pathway activation in the absence of prior therapy. The patients ranged in age from 28 to 88 with a median age of 58 (Table 1); all
25 were diagnosed with a GBM on biopsy by at lease two independent Neuropathologists.

[0117] PTEN protein expression was diminished or lost in 17/45 GBMs (38%) (Fig. 1, Table 2). This is in agreement with previous studies that have used DNA-based methods to detect PTEN loss in 30-40% of GBMs (see, e.g., Liu et al., Cancer Res. *57:* 5254-7., 1997; Schmidt et al., J Neuropathol Exp Neurol. *58:* 1170-83., 1999; Smith et al.,

J Natl Cancer Inst. *93:* 1246-56., 2001). Akt phosphorylation was significantly associated with diminished PTEN immunohistochemical expression ($p<0.00001$) (Fig 1., Table 2). PTEN loss was not significantly associated with expression of p-Erk (Table 2), whose activation is independent of PI3'K/Akt signaling. To determine whether Akt activation
5 correlated with concurrent activation of downstream effectors, we used phosphorylation specific antibodies directed against mTOR, FKHR and S6. mTOR and FKHR are directly phosphorylated by Akt (see, e.g., Vivanco et al., Nat Rev Cancer. *2:* 489-501., 2002; Hidalgo et al., Oncogene. *19:* 6680-6686., 2000); S6 is phosphorylated by p70 S6 kinase, which is itself a target of Akt (see, e.g., Blume-Jensen et al., Nature. *411:* 355-365.,
10 2001). Akt activation was significantly associated with expression of p-mTOR ($p=0.04$) and p-FKHR ($p=0.006$)(Table 3). Akt activation was also correlated with strong S6 phosphorylation (2+) ($p=0.001$), although weaker S6 phosphorylation (1+) was also detected in Akt- negative tumors (Table 3). This latter result is not surprising considering that S6 can be activated by Erk in a PI3'K/Akt independent fashion (see, e.g., Iijima et
15 al., J Biol Chem. *277:* 23065-75., 2002; Shi et al., J Biol Chem. *277:* 15712-20., 2002). Taken together, these results provides evidence that PI3'K/Akt pathway activation can be detected in routinely processed paraffin-embedded biopsy samples, and demonstrate that PTEN protein loss is associated with PI3'K/Akt pathway activation in GBMs.

20 **EXAMPLE 6: Akt pathway activation in GBMs lacking PTEN protein loss: assessment of EGFR/EGFRvIII-mediated signaling**

[0118] PTEN loss did not appear to be the only route to Akt activation; expression of p-Akt and downstream effectors p-mTOR, p-FKHR and p-S6 was also
25 detected in 28% of GBMs with no immunohistochemical PTEN loss (Table 2). Because the PI3'K/Akt pathway can be activated by EGFR signaling, we analyzed EGFR and EGFRvIII expression and assessed their association with PI3'K/Akt pathway activation in the setting of normal PTEN immunohistochemical staining. EGFR immunopositivity was detected in 60% of GBMs (Fig. 2), in line with previous reports (see, e.g., Smith et
30 al., J Natl Cancer Inst. *93:* 1246-56., 2001; Watanabe et al., Brain Pathol. *6:* 217-23; discussion 23-4., 1996; Ekstrand et al., Proc Natl Acad Sci U S A. *89:* 4309-13., 1992;

Frederick et al., Cancer Res. *60:* 1383-7., 2000; Hayashi et al., Brain Pathol. *7:* 871-5., 1997; Nagane et al., Cancer Lett. *162 Suppl:* S17-S21., 2001; Nishikawa et al., Proc Natl Acad Sci U S A. *91:* 7727-31., 1994; Wikstrand et al., Cancer Res. *57:* 4130-40., 1997). Immunohistochemical expression of the constitutively active mutant EGFRvIII was detected in 56% of EGFR positive tumors (44% of tumors overall) (Fig. 2) (Smith et al., J Natl Cancer Inst. *93:* 1246-56., 2001; Nagane et al., Cancer Lett. *162 Suppl:* S17-S21., 2001; Nishikawa et al., Proc Natl Acad Sci U S A. *91:* 7727-31., 1994; Wikstrand et al., Cancer Res. *57:* 4130-40., 1997). Strongly activated Akt (2+ staining) was not detectable in GBMs with normal PTEN immunohistochemical expression that lacked EGFR and EGFRvIII expression (Table 2). In contrast, 36% of GBMs with normal PTEN immunohistochemical expression that also co-expressed EGFR and EGFRvIII stained strongly for activated Akt (p=0.06) (Table 2). Although the subset of tumors was small, co-expression of EGFRvIII along with EGFR appeared to be required for strong Akt activation (2+ staining)(Table 2). These results provides evidence that co-expression of EGFR and EGFRvIII can promote Akt activation in GBMs with normal PTEN protein expression. Consistent with this, downstream activation of mTOR, S6 and FKHR were also significantly more likely to be strongly activated (2+ staining) in GBMs with normal PTEN expression when EGFR and EGFRvIII were co-expressed ($p<0.002$).

[0119] In addition to the Akt pathway, EGFR, and EGFRvIII can also activate Erk. Therefore, we determined whether Erk phosphorylation was associated with EGFR and EGFRvIII expression. Overall, Erk phosphorylation was detected in 51% of GBMs. More importantly, expression of phosphorylated Erk was significantly associated with EGFR expression (p=0.007)(Fig. 2; Table 4). Phosphorylated Erk was expressed in 75% of EGFR+/EGFRvIII negative GBMs and 88% of EGFR+/EGFRvIII positive GBMs.

EXAMPLE 7: Prognostic implications of Akt and Erk pathway activation

[0120] Previous studies have not shown a clear prognostic implications for PTEN loss, EGFR over-expression or EGFRvIII expression in GBMs. In line with this, we found no statistically significant association between PTEN protein loss, EGFR or EGFRvIII expression and either time to progression or overall survival. In contrast,

coordinate pathway activation appeared to have prognostic implications. Expression of p-Akt was not significantly associated with survival or progression. In contrast, activation of the downstream pathway, as detected by concurrent phosphorylation of mTOR, FKHR and S6, was significantly associated with both a shorter time to progression (p=0.002) and a decreased overall survival (p=0.02). This finding may reflect a contribution from additional inputs downstream of Akt, such as Erk-mediated activation of S6 kinase and nutrient-mediated activation of mTOR. Alternatively, this panel of three phospho-specific antibodies may be a more sensitive method to detect Akt pathway activation than a single phospho-Akt antibody alone. Erk activation was also significantly associated with more rapid progression and diminished overall survival in this subset of primary GBM patients (<0.04) (Table 5). these findings are the first demonstration that pathway activation has an impact on tumor progression in GBM patients.

EXAMPLE 8: Kinase Inhibitors Akt and Erk pathway activation

[0121] Figures 3A and 3B provide an illustration of the interaction between members of the PI3K/Akt pathway and kinase inhibitors.

[0122] Figure 3A shows that rapamycin inhibits S6 phosphorylation in glioblastoma in vivo. In particular, Figure 3A provides data from an analysis of a cohort of patients on a rapamycin clinical trial. This data shows that a substantial reduction in S6 phosphorylation relative to the initial biopsy was detected in the tumor in the majority of patients treated with rapamycin for 5 days prior to undergoing surgical resection. Control patients showed a uniformly high level of S6 phosphorylation. This data provides evidence that rapamycin inhibited mTOR signaling at the level of S6 phosphorylation in the majority of glioblastoma patients. In addition, this data illustrates how the detection of pathway activation by immunohistochemistry (IHC) correlates with detection by western blotting.

[0123] Figure 3B shows that the rapamycin-mediated inhibition of S6 phosphorylation correlates with diminished tumor proliferation. In this Figure, Ki-67, a marker of cellular proliferation was used to assess whether rapamycin-mediated inhibition of S6 had an effect on tumor growth. This data provides evidence that the

rapamycin-mediated inhibition of mTOR signaling at the level of S6 phosphorylation correlated with diminished tumor cell proliferation.

EXAMPLE 9: Antibody based profiling of the PI3K pathway in clinical prostate cancer.

[0124] The following example illustrates the use of the methods and materials of the invention disclosed herein on prostate cancer samples. As noted above, constitutive activation of the PI3K pathway and its downstream effectors, as a result of PTEN loss or by other mechanisms, occurs in a high proportion of cancers such as prostate cancers, making it an ideal template for the design of clinical trials involving PI3K pathway inhibitors. Prostate cancers also present unique organ specific challenges, in that tumors are heterogeneous and the amount of diagnostic tissue is extremely limited. Working within these parameters, we disclose a set of immunohistochemical assays that define activation of the PI3K pathway in clinical samples. Using both univariate and multivariate analyses, we show that loss of PTEN is highly correlated with activation of AKT and this, in turn is associated with the phosphorylation of S6, one of its main effectors. Three antibodies directed at these targets define a molecular signature of PTEN loss and/or AKT pathway activation in prostate cancer.

[0125] We have previously shown that PTEN deficient prostate cancer cell lines and xenografts are more sensitive to the pharmacologic inhibition of mTOR using the rapamycin analogue, CCI-779, when compared to their wild type counterparts (Neshat et al., Proc Natl Acad Sci U S A, 98: 10314-10319, 2001; Podsypanina et al., Proc Natl Acad Sci U S A, 98: 10320-10325, 2001). We also demonstrated that the phosphorylation of S6 ribosomal protein (hereafter called S6) was a potential surrogate marker for both pathway activation and mTOR inhibition by drug.

[0126] Lessons learned from molecularly targeted therapies point to two key elements that will ultimately determine how to efficiently translate these findings into the clinic (Druker et al., N Engl J Med, 344: 1031-1037, 2001; Druker et al., N Engl J Med, 344: 1038-1042, 2001; Bianco et al., Oncogene, 22: 2812-2822, 2003; Brognard et al., Cancer Res, 61: 3986-3997, 2001). One, we need to identify subsets of patients who will

most likely to benefit from this class of drugs. The importance of this knowledge is exemplified by the recent experience with EGFR inhibitors in lung cancer, where knowing the mutation status of the EGFR can enrich for patients most likely to benefit from these class of drugs (Paez et al., Science, 304: 1497-1500, 2004; Lynch et al., N Engl J Med, 350: 2129-2139, 2004). Our current understanding suggests this may depend on the level of signal activation of the pathway being targeted and the molecular lesion leading to target activation. Second, we need to measure inhibition of the signaling pathway when using targeted therapy to guide dose selection and scheduling. Translating these goals to the clinic presents several challenges not encountered *in vitro*. Diagnostic needle core biopsies of the prostate invariably consist of small amounts of tissue. Also, unlike cancer cell lines, clinical samples of human prostate cancers are not homogenous populations, but consist instead of a mixture of both normal and tumor bearing cells. Clinical decisions using molecularly targeted agents are likely to require assessment of signaling pathway abnormalities using limited biopsy material.

[0127] With these goals in mind, this Example illustrates the detection of activated signaling pathways *in situ* on formalin fixed, paraffin embedded tissue, analogous to detecting Her-2/Neu amplification by immunohistochemistry in breast cancers. Since the target (mTOR) is a kinase, we examined the phosphorylation status of its downstream effectors by utilizing activation specific, phospho-specific antibodies. When examined within the context of a tissue microarray, we showed that PTEN loss was highly correlated with AKT activation, and AKT activation was correlated with upregulation of p-S6.

MATERIALS AND METHODS

Human prostate cancer tissue microarray

[0128] We constructed a tissue microarray from formalin fixed, paraffin embedded archival tissue blocks, from 133 radical prostatectomies, performed for prostate cancer. The distribution of Gleason scores and Pathological TNM stage were as follows: 79 cases had Gleason score 5-6; 37 cases Gleason score 7 and 9 cases Gleason score 8-10. Eighty four cases were organ confined (i.e. Stage 2), 34 were Stage 3 and 13

cases were Stage 4. The array was designed so that three cores were taken from tumor bearing tissue and one core from morphologically benign tissue (only areas containing normal prostate glands and stroma were sampled; atrophic and hyperplastic glands were not included in the array construction or in the evaluation), from the same patient. These
5 cores were placed adjacent to each other on the tissue array, enabling comparison of antibody staining pattern and intensity between benign and cancer tissue from the same patient.

***In vitro* optimization of phospho-specific antibodies**

10 **[0129]** Cells (LNCaP, DU 145, HT 129) were grown in culture (DMEM supplemented with 10% FBS, L-Glutamine, Penicillin and Streptomycin) and exposed to vehicle, the PI3K inhibitor LY294002 (30μM), the mTOR inhibitor, CCI-779 (20nM) for 18 hours or the MEK inhibitor, UO126 (20μM) combined with 100ng/ml of EGF for 20 minutes. Cell pellets were prepared, formalin fixed and embedded in paraffin, then
15 cut for immunohistochemical staining with phospho-AKT, -S6, -FKHR and -ERK antibodies. We performed antigen retrieval with a 10mM sodium citrate buffer (pH 6.0) for 30 minutes in a pressure cooker. Peroxidase activity was quenched with 3% hydrogen peroxide in water. Sections were stained overnight at 4^0C with monoclonal antibodies to PTEN at 1:300 (clone 6H2.1; Cascade Bioscience, Winchester, MA. This
20 antibody recognizes a 99 amino acid epitope at the C-terminus of PTEN) and polyclonal phosphorylation-specific antibodies to p-AKT (Ser 473), p-FKHR which recognizes phosphorylated forms of the forkhead family of transcription factors FKHR (FOXO 1), FKHRL1 (FOXO 3a) and AFX (FOXO 4), p-S6 Ribosomal protein (Ser 235/236), p-ERK (Thr-202/Tyr-204) at 1:100 (Cell Signaling Technology, Beverly, MA). This was
25 followed by application of biotinylated secondary antibodies (Vector) at 1:1000 dilution for 30 minutes and Avidin-biotin complex (Elite ABC; Vector). Negative control slides received normal mouse serum (DAKO) as the primary antibody. Diaminobenzidine tetrahydrochloride (DAB) was used as the enzyme substrate to visualize specific antibody localization for PTEN and p-AKT; Vector NovaRed (Vector) for p-FKHR, p-S6 and p-
30 ERK. Slides were counterstained with Harris hematoxylin. For immunofluorescence

assays, post fixation in paraformaldehyde, the cells were probed with Total AKT (1:50) and p-AKT (1:250); the secondary antibodies Alexa Flour anti-rabbit and anti-mouse (1:1000) [Molecular Probes, Eugene, Oregon]. Following incubation, images were captured on a Nikon fluorescence microscope and then overlaid using Adobe Photoshop 5 software.

Scoring protocols

PTEN

[0130] PTEN staining was scored according to a previously established scale of 0-2, which has been shown to be highly consistent (Choe et al., Cancer Res, 63: 2742-10 2746, 2003; Gimm et al., Am J Pathol, 156: 1693-1700, 2000; Perren et al., Am J Pathol, 155: 1253-1260, 1999; Zhou et al., Am J Pathol, 157: 1123-1128, 2000). Tumor cells are graded as 2 if their staining intensity is equal to that of the adjacent benign cells, graded as 1 if their staining was diminished relative to the benign cells and 0 if staining intensity is undetectable in the tumor cells and is present in the benign cells. Only cores 15 containing tumor and that were technically interpretable (e.g. immunoreactivity in the benign glands) were included in the scoring protocol. The arrays were scored by one genitourinary pathologist (G.V.T) on two occasions. In addition, twenty percent of the cases were randomly picked and scored by a second genitourinary pathologist in an independent manner, i.e. with no prior knowledge of the original results. Both inter-rater 20 and intra-rater agreement were >90%.

Phosphorylation specific antibodies

[0131] For scoring purposes, we only included membranous or cytoplasmic staining for p-AKT and only cytoplasmic staining for p-FKHR and p-S6. p-AKT, p-S6 25 and p-FKHR were scored according to a scale of 0-2 (0=no staining; 1=mild intensity of cytoplasmic staining and 2=strong cytoplasmic staining). For p-AKT and p-S6, staining of 1 and 2 were considered positive; for p-S6, staining of 2 was considered positive, as previously published (Choe et al., Cancer Res, 63: 2742-2746, 2003). For p-ERK, tumors that focally contained greater than 5% positive nuclear staining were considered positive,

as previously reported (Choe et al., Cancer Res, 63: 2742-2746, 2003; Mukohara et al., Lung Cancer, 41: 123-130, 2003). The agreement between the pathologists (see PTEN scoring protocol) as well as the same pathologist on independent reviews was 85% for p-AKT and p-S6K; 80% for p-FKHR and 90% for p-ERK.

5 **Statistical analysis of immunohistochemical scoring**

[0132] When studying the correlations between the staining scores, we used Pearson correlation, which is appropriate for ordinally scored data (Table 7). When fitting a logistic regression model where a staining score was the outcome, we dichotomized the staining score (e.g. 0 vs. 1, 2), i.e. no expression versus at least some
10 expression. The computation of the Pearson correlations and the logistic regression analysis were all carried with the R software (http://cran.r-project.org/) (see, e.g. Ihaka et al., J.Comput.Graphical Statistics, 5: 299-314, 1996). We then fitted two multivariate logistic regression models (Table 8). First we regressed PTEN status on p-AKT, p-FKHR and p-S6. Second we regressed p-AKT status on the p-FKHR family and p-S6K.
15 **[0133]** To depict the relationship between the variables, we used the R function cmd scale to arrive at a two-dimensional classical multidimensional scaling plot (Figure 7). The aim of the MDS is to represent dissimilarities (here, one minus the squared Pearson correlation) between points (here, biomarker staining scores) well by choosing a two-dimensional configuration of points that minimizes a "stress" function (Cox et al.,
20 Multidimensional Scaling. United Kingdom: CRC Press, 2001).

Characterization of PTEN and the phospho-specific antibodies

[0134] First, we characterized the specificity of the PTEN antibody used in our assays by probing cell lines with known endogenous PTEN expression. LNCaP cells, which have a deletion of one PTEN allele and a mutation of the other allele stained
25 negatively while DU145 cells, which are PTEN wild, stained positively (**Figure 5A**).

[0135] To validate the specificity of the phospho-specific antibodies, we used several confirmatory assays, i.e. preincubation with specific antigen peptides, immunofluorescence and treatment with pharmacological inhibitors of the pathway. We first examined the specificity of the antibodies when preincubated with a specific antigen

peptide. Serial sections of a formalin fixed, paraffin embedded breast tumor were stained with p-AKT, p-S6, p-FKHR and p-ERK singly and then in combination with a specific antigen peptide. The phospho-specific staining was blocked when preincubated with its antigen peptide, confirming its specificity (**Figure 5B**). Next, we examined the relationship between Total AKT and p-AKT by immunofluorescence. LNCaP cell pellets were probed with FITC labeled Total AKT (red) and p-AKT (green). LNCaP cells have loss of PTEN, with subsequent activation of AKT, as seen by the co-localization of AKT and p-AKT (yellow). When treated with the PI3K inhibitor LY294002, a decrease in the signal intensity was noted, consistent with known effects of this PI3K inhibitor (**Figure 5C**). Next, we asked if LY294002 was able to block the activation of the signaling molecules downstream of PIP3. Addition of LY294002 successfully blocked the activation of AKT as well as S6, as seen by the decrease in staining intensity pre and post treatment. We noted that the staining intensity of p-FKHR in the untreated cells was weaker compared to AKT and S6 but that this was completely abolished by the addition of LY294002. Addition of the PI3K inhibitor appeared to have no effect on the staining intensity of p-ERK (**Figure 5D**). We proceeded to examine the staining characteristics of p-ERK in HT129 cells, by inducing its expression by the addition of EGF alone (left panel), or in combination with a MEK inhibitor, UO-126. Addition of UO-126 to the induced cells resulted in decreased staining, consistent with *in vitro* signaling data (right panel).

[0136] Next we asked whether the phospho-specific antibodies could be used to monitor treatment effect. We probed LNCaP cells that had been treated with either CCI-779, an inhibitor of mTOR, with p-AKT and p-S6. As expected, pharmacological inhibition of m TOR resulted in downregulation of S6 phosphorylation but had no effect on p-AKT (**Figure 5E**). This result raises the possibility of using these phospho-specific antibodies as tools to monitor target inhibition in patients treated with mTOR inhibitors.

Defining the loss of PTEN protein expression *in vivo* and correlation with signaling pathway upregulation

[0137] Having validated the performance characteristics of these antibodies in defined *in vitro* cell line systems, we next examined their immunohistochemical staining

profiles within the tissue microarray. First, we defined the frequency of PTEN loss as measured by protein expression using an antibody to the C-terminus. [The C-terminal tail region contains PDZ domain binding sequences and multiple phosphorylation sites. We and others have shown that mutations in the C-terminal tail of PTEN results in loss of protein stability as well as decrease in membrane affinity (Wu et al., Proc Natl Acad Sci U S A, 97: 4233-4238, 2000; Torres et al., J Biol Chem, 276: 993-998, 2001; Das et al., Proc Natl Acad Sci U S A, 100: 7491-7496, 2003). Loss of PTEN staining with this antibody should reflect impaired PTEN activity]. Since some cancers may have missense mutations in PTEN that do not alter protein expression, we recognize that this approach may underestimate the true frequency of PTEN loss and function. Of note, immunohistochemical assays will identify loss of protein expression in tumors with homozygous deletions, nonsense mutations, certain internal deletions, promoter methylation and finally posttranscriptional modifications. Complete loss of PTEN staining was seen in 29% (score=0; n=37); decreased staining compared to benign in 11% (Score =1; n= 14), and staining intensity in tumor equivalent to that in benign glands in 60% (score =2; n= 78; **Figure 6A**). This is in keeping with previous reports of absent PTEN staining, performed on conventional tissue sections as well as tissue microarrays (McMenamin et al., Cancer Res, 59: 4291-4296, 1999; Halvorsen et al., Clin Cancer Res, 9: 1474-1479, 2003).

[0138] PTEN loss was significantly inversely correlated with activation of p-AKT ($r = -0.46$, $P = 0.00000002$) but not p-ERK **(Figure 6B).** Activation of p-AKT (Scheid et al., Mol Cell Biol, 22: 6247-6260, 2002) was significantly correlated with S6 phosphorylation ($r = 0.21$, $P= 0.01$) but not p-FKHR. Both AKT and S6 phosphorylation did not correlate with p-ERK, a target of the MAPK pathway (**Table 7**).

[0139] We next examined PTEN loss within a multivariate logistic regression model, with PTEN as the outcome and p-AKT, p-FKHR and p-S6 as covariates (**Table 8**). A strong independent association between PTEN and p-AKT was identified ($P= 0.0005$), with the odds of a case being PTEN positive when p-AKT is positive is only 0.28 (95% CI, 0.14-0.58) that of a p-AKT negative case. Fitting the model for p-AKT

and including p-FKHR and p-S6 as covariates demonstrated a significant independent association between AKT and p-S6 activation (P=0.028). The odds of a case being p-AKT positive when p-S6 is phosphorylated is 2.17 times (95%CI, 1.09-4.32) that of a case with p-S6 negative and AKT (positive vs. negative). The association between PTEN 5 loss and p-S6 activation almost reached statistical significance (P=0.065).

[0140] We then asked if our data might be used to uncover relationships between these relationships without prior knowledge of the connectivity between these proteins using MDS analysis, a form of principal component analysis. MDS is an unsupervised data analysis method that allows examination of potential relationships 10 between variables without assuming previous knowledge of their interaction. Using this approach, the signaling molecules are plotted in two dimensions and the distance between two molecules provide a measure of their inter-relatedness (Venables et al., Modern Applied Statistics with S-Plus. New York: Springer, 1999). This plot is a graphical representation of the correlations found in Table 7. Loss of PTEN protein 15 expression and AKT activation are closely related, as shown by the short distance between them. In contrast, p-ERK is distant from PTEN and p-AKT (**Figure 7**).

[0141] As shown above, utilizing prostate cancer tissue microarrays, we have successfully shown that this pathway can be interrogated *in vivo*, using immunohistochemical assays. We have identified a subset of patients who exhibit 20 complete loss of PTEN protein expression. Absence of staining (i.e. negative staining), when viewed in conjunction with the appropriate controls is a firm, objective read-out and is readily reproducible across centers, analogous to the current standard of clinical care, where loss of basal cell marker expression (i.e. absent immunohistochemical staining for the high molecular weight keratin-903 and/or p63) is widely used as part of 25 the diagnostic criteria to distinguish between benign as well as in-situ lesions and prostate cancer (Wojno et al., Am J Surg Pathol, 19: 251-260, 1995; Signoretti et al., Am J Pathol, 157: 1769-1775, 2000; Weinstein et al., Mod Pathol, 15: 1302-1308, 2002; Zhou et al., Am J Surg Pathol, 27: 365-371, 2003).

[0142] Loss of PTEN protein expression was significantly correlated with 30 activation of AKT. Activation of AKT was in turn, associated with phosphorylation of

S6, in keeping with what is known of the relationships between these signaling molecules(Luo et al., Cancer Cell, 4: 257-262, 2003; Schmelzle et al., Cell, 103: 253-262, 2000; Paez et al., Cancer Treat Res, 115: 145-167, 2003; Chou et al., Cell, 85: 573-583, 1996; Berven et al., Exp Cell Res, 296: 183-195, 2004). In terms of PTEN biology, it has been shown in genetic systems (i.e. conditional knock-outs) that the dosage of PTEN loss correlates with murine prostate cancer progression (Wang et al., Cancer Cell, 4: 209-221, 2003; Trotman et al., PLoS Biol, 1: E59, 2003). In our current series, PTEN loss, when examined by Pearson and Multivariate analysis, is significantly associated with phosphorylation of AKT. Conceptually, it may be that PTEN score 1 may reflect decreased (but not absent) gene activity.

[0143] The lack of correlation between p-AKT and p-FKHR requires further examination, especially in light of previously published work from our institution showing a significant association in glioblastomas (Choe et al., Cancer Res, 63: 2742-2746, 2003). When we were developing protocols to optimize the phospho-specific antibody immunohistochemical assays, we noted that the staining intensity of p-FKHR was less intense when compared to p-AKT or p-S6. This variation in staining characteristics, when transferred to human samples of different fixation and processing methodologies may lessen the likelihood of consistent, reproducible results. Of note, p-FKHR scored the lowest in the intra and inter-observer correlation percentiles, suggesting that subtle differences in staining intensity may lead to variations in the scoring protocol. p-ERK is not correlated with any molecules in this pathway, consistent with in vitro data that places the MAPK pathway parallel to, rather than epistatic to the PI3K cascade.

[0144] Using these phospho-specific antibodies, we were also able to follow changes in phosphorylation status of these proteins in a prostate cancer cell line, before and after treatment with CCI-779. This supports utility of these antibodies to confirm target inhibition in patient samples post treatment.

[0145] We did not see any correlation between PTEN status (or any of the other markers) and Gleason Score or the pathological stage. In contrast, two other studies have found associations between loss of PTEN protein expression or increased

activation of AKT with higher Gleason scores and advanced stage (McMenamin et al., Cancer Res, 59: 4291-4296, 1999; Malik et al., Clin Cancer Res, 8: 1168-1171, 2002). One potential explanation is that our dataset may be underpowered to address this question, as it is populated by lower Gleason grades and pathologic stages. In future studies, it will
5 be important to correlate PI3K pathway activation with clinical outcome.

[0146] From a clinical standpoint, it will be important to determine if the molecular aberrations seen in the tissue microarray cores can be reproduced in the context of a clinical trial where core needle biopsies might be used to guide treatment decisions with a targeted agent. Ultimately, these questions can only be accurately
10 answered by examining the staining patterns in matched biopsy and radical prostatectomy specimens from the same patients, as we have done previously with other biomarkers such as p27 (Thomas et al., J Urol, 164: 1987-1991, 2000). Indeed, these results form the basis of multicenter, neoadjuvant clinical trial of an mTOR inhibitor in men with newly diagnosed, high-risk prostate cancer. Ultimately, it will be of interest to
15 compare data such as this, obtained by immunohistochemical assays, with genomic data obtained by PTEN gene sequencing or LOH analysis from clinical material. At present, such techniques require that samples contain a high fraction of tumor cells (vs. normal) or isolation of pure populations of tumor cells by laser capture microdissection, thereby limiting their current use in the clinic.

20

[0147] The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the invention, and any that are functionally equivalent are within the scope of the invention. Various modifications to the models and methods of the invention, in addition to those
25 described herein, will become apparent to those skilled in the art from the foregoing description and teachings, and are similarly intended to fall within the scope of the invention. Such modifications or other embodiments can be practiced without departing from the true scope and spirit of the invention.

TABLES

Table 1 Patient Characteristics

Clinical Characteristics	#
Sex	
M	29
F	16
Age (years)	
Median	58
Mean	58
Range	28-88
Time to progression (days)	
Median	183
Mean	227
Range	54-1006
Survival (days)	
Median	412
Mean	427
Range	86-1794

5

Table 2 Association between PTEN expression and Akt pathway activation

			p-Akt	p-mTOR			p-FKHR		p-S6			p-ERK		
	0	1	2	0	1	2	0	1	2	0	1	2	+	-
PTEN-	2	1	13	3	4	10	2	2	12	4	3	10	8	7
PTEN+	18	3	4	6	8	11	10	2	13	5	7	12	16	8
p-value			0.00001			ns			ns			ns		ns
PTEN+/EGFR-/EGFRvIII-	6	2	0	5	2	0	7	0	1	5	2	0	nd	nd
PTEN+/EGFR+/EGFRvIII-	3	1	0	0	1	2	1	2	1	0	1	3	nd	nd
PTEN+/EGFR+/EGFRvIII+	7	0	4	0	3	9	2	1	10	0	3	10	nd	nd
p-value			0.06			0.001			0.002			0.001		nd

10

Table 3 Association between Akt activation and downstream signaling

	p-mTOR 0	p-mTOR 1	p-mTOR 2	p-value	p-FKHR 0	p-FKHR 1	p-FKHR 2	p-value	p-S6 0	p-S6 1	p-S6 2	p-value
p-Akt-	6	7	7		10	1	9		6	7	7	
p-Akt+	1	5	14	0.04	1	3	16	0.006	4	3	13	0.15 (*0.003)

5

Table 4 Univariate analysis between EGFR receptor status and downstream signalling.

10

	Pearson Correlation	p-value
EGFR		
EGFRvIII	0.31	0.04
p-Erk	0.34	0.03
p-Akt	0.07	0.67
p-FKHR	0.25	0.12
p-mTOR	0.24	0.13
p-S6	0.3	0.06
EGFRvIII		
p-FKHR	0.33	0.04
p-mTOR	0.31	0.06
p-S6	0.3	0.06

Table 5 Univariate association between pathway activation and prognosis

	Time to progression (days)	p-value	Survival (days)	p-value
p-Erk+	148		356	
p-Erk-	263	0.05	488	0.02
p-mTOR+/p-FKHR+/p-S6+	142	0.002	357	0.02
p-mTOR-/p-FKHR-/p-S6-	308		528	
p-Akt+	176	NS	358	NS
p-Akt-	238		419	
PTEN +	153	NS	438	NS
PTEN -	212		346	
EGFR+	151	NS	385	NS
EGFR-	243		412	
EGFRvIII+	135	NS	420	NS
EGFRvIII-	230		351	

5

TABLE 6 POLYPEPTIDE SEQUENCES

For convenience, Table 6 provides the sequences, accession numbers and illustrative references for the
10 well known polypeptides discussed herein. In certain sequences in this Table, illustrative residues that are typically phosphorylated during pathway signalling are shown in boldface type.

15 **S6 (NP 001001, gi:17158044) 249 amino acids**
See, e.g. Pata et al., Gene 121 (2), 387-392 (1992)

MKLNISFPATGCQKLIEVDDERKLRTFYEKRMATEVAADALGEEWKGYVVRISGGND
KQGFPMKQGVLTHGRVRLLLSKGHSCYRPRRTGERKRKSVRGCIVDANLSVLNLVIV
KKGEKDIPGLTDTTVPRRLGPKRASRIRKLFNLSKEDDVRQYVVRKPLNKEGKKPRT
20 KAPKIQRLVTPRVLQHKRRRIALKKQRTKKNKEEAAEYAKLLAKRMKEAKEKRQEQI
AKRRRL**SS**LRASTSKSESSQK (SEQ ID NO: 1)

m-TOR (NP 004949, gi:4826730) 2549 amino acids

See, e.g. Brown et al., Nature 369 (6483), 756-758 (1994)

MLGTGPAAATTAATTSSNVSVLQQFASGLKSRNEETRAKAAKELQHYVTMELREMSQ
5 EESTRFYDQLNHHIFELVSSSDANERKGGILAIASLIGVEGGNATRIGRFANYLRNL
LPSNDPVVMEMASKAIGRLAMAGDTFTAEYVEFEVKRALEWLGADRNEGRRHAAVLV
LRELAISVPTFFFQQVQPFFDNIFVAVWDPKQAIREGAVAALRACLILTTQREPKEM
QKPQWYRHTFEEAEKGFDETLAKEKGMNRDDRIHGALLILNELVRISSMEGERLREE
MEEITQQQLVHDKYCKDLMGFGTKPRHITPFTSFQAVQPQQSNALVGLLGYSSHQGL
10 MGFGTSPSPAKSTLVESRCCRDLMEEKFDQVCQWVLKCRNSKNSLIQMTILNLLPRL
AAFRPSAFTDTQYLQDTMNHVLSCVKKEKERTAAFQALGLLSVAVRSEFKVYLPRVL
DIIRAALPPKDFAHKRQKAMQVDATVFTCISMLARAMGPGIQQDIKELLEPMLAVGL
SPALTAVLYDLSRQIPQLKKDIQDGLLKMLSLVLMHKPLRHPGMPKGLAHQLASPGL
TTLPEASDVGSITLALRTLGSFEFEGHSLTQFVRHCADHFLNSEHKEIRMEAARTCS
15 RLLTPSIHLISGHAHVVSQTAVQVVADVLSKLLVVGITDPDPDIRYCVLASLDERFD
AHLAQAENLQALFVALNDQVFEIRELAICTVGRLSSMNPAFVMPFLRKMLIQILTEL
EHSGIGRIKEQSARMLGHLVSNAPRLIRPYMEPILKALILKLKDPDPDPNPGVINNV
LATIGELAQVSGLEMRKWVDELFIIIMDMLQDSSLLAKRQVALWTLGQLVASTGYVV
EPYRKYPTLLEVLLNFLKTEQNQGTRREAIRVLGLLGALDPYKHKVNIGMIDQSRDA
20 SAVSLSESKSSQDSSDYSTSEMLVNMGNLPLDEFYPAVSMVALMRIFRDQSLSHHHT
MVVQAITFIFKSLGLKCVQFLPQVMPTFLNVIRVCDGAIREFLFQQLGMLVSFVKSH
IRPYMDEIVTLMREFWVMNTSIQSTIILLIEQIVVALGGEFKLYLPQLIPHMLRVFM
HDNSPGRIVSIKLLAAIQLFGANLDDYLHLLLPPIVKLFDAPEAPLPSRKAALETVD
RLTESLDFTDYASRIIHPIVRTLDQSPELRSTAMDTLSSLVFQLGKKYQIFIPMVNK
25 VLVRHRINHQRYDVLICRIVKGYTLADEEEDPLIYQHRMLRSGQGDALASGPVETGP
MKKLHVSTINLQKAWGAARRVSKDDWLEWLRRLSLELLKDSSSPSLRSCWALAQAYN
PMARDLFNAAFVSCWSELNEDQQDELIRSIELALTSQDIAEVTQTLLNLAEFMEHSD
KGPLPLRDDNGIVLLGERAAKCRAYAKALHYKELEFQKGPTPAILESLISINNKLQQ
PEAAAGVLEYAMKHFGELEIQATWYEKLHEWEDALVAYDKKMDTNKDDPELMLGRMR
30 CLEALGEWGQLHQQCCEKWTLVNDETQAKMARMAAAAAWGLGQWDSMEEYTCMIPRD
THDGAFYRAVLALHQDLFSLAQQCIDKARDLLDAELTAMAGESYSRAYGAMVSCHML
SELEEVIQYKLVPERREIIRQIWWERLQGCQRIVEDWQKILMVRSLVVSPHEDMRTW
LKYASLCGKSGRLALAHKTLVLLLGVDPSRQLDHPLPTVHPQVTYAYMKNMWKSARK
IDAFQHMQHFVQTMQQQAQHAIATEDQQHKQELHKLMARCFLKLGEWQLNLQGINES
35 TIPKVLQYYSAATEHDRSWYKAWHAWAVMNFEAVLHYKHQNQARDEKKKLRHASGAN
ITNATTAATTAATATTTASTEGSNSESEAESTENSPTPSPLQKKVTEDLSKTLLMYT
VPAVQGFFRSISLSRGNNLQDTLRVLTLWFDYGHWPDVNEALVEGVKAIQIDTWLQV
IPQLIARIDTPRPLVGRLIHQLLTDIGRYHPQALIYPLTVASKSTTTARHNAANKIL
KNMCEHSNTLVQQAMMVSEELIRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLE
40 PLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFR
RISKQLPQLTSLELQYVSPKLLMCRDLELAVPGTYDPNQPIIRIQSIAPSLQVITSK
QRPRKLTLMGSNGHEFVFLLKGHEDLRQDERVMQLFGLVNTLLANDPTSLRKNLSIQ
RYAVIPLSTNSGLIGWVPHCDTLHALIRDYREKKKILLNIEHRIMLRMAPDYDHLTL
MQKVEVFEHAVNNTAGDDLAKLLWLKSPSSEVWFDRRTNYTRSLAVMSMVGYILGLG
45 DRHPSNLMLDRLSGKILHIDFGDCFEVAMTREKFPEKIPFRLTRMLTNAMEVTGLDG
NYRITCHTVMEVLREHKDSVMAVLEAFVYDPLLNWRLMDTNTKGNKRSRTRTDSYSA

GQSVEILDGVELGEPAHKKTGTTVPESIH**S**FIGDGLVKPEALNKKAIQIINRVRDKL
TGRDFSHDDTLDVPTQVELLIKQATSHENLCQCYIGWCPFW (SEQ ID NO: 2)

FORKHEAD (NP 002006, gi:9257222) 655 amino acids

See, e.g. Anderson et al., Genomics 47 (2), 187-199 (1998)

MAEAPQVVEIDPDFEPLPRPRSC**T**WPLPRPEFSQSNSATSSPAPSGSAAANPDAAAG
LPSASAAAVSADFMSNLSLLEESEDFPQAPGSVAAAVAAAAAAAATGGLCGDFQGPE
AGCLHPAPPQPPPPGPLSQHPPVPPAAAGPLAGQPRKSSSSRRNAWGNLSYADLITK
AIESSAEKRLTLSQIYEWMVKSVPYFKDKGDSNSSAGWKNSIRHNLSLHSKFIRVQN
EGTGKSSWWMLNPEGGKSGKSPRRRAASMDNNSKFAKSRSRAAKKKASLQSGQEGAG
DSPGSQFSKWPASPGSHSNDDFDNWSTFRPRTSSNASTISGRLSPIMTEQDDLGEGD
VHSMVYPPSAAKMASTLPSLSEISNPENMENLLDNLNLLSSPTSLTVSTQSSPGTMM
QQTPCYSFAPPNTSLNSPSPNYQKYTYGQSSMSPLPQMPIQTLQDNKSSYGGMSQYN
CAPGLLKELLTSDSPPHNDIMTPVDPGVAQPNSRVLGQNVMMGPNSVMSTYGSQASH
NKMMNPSSHTHPGHAQQTSAVNGRPLPHTVSTMPHTSGMNRLTQVKTPVQVPLPHPM
QMSALGGYSSVSSCNGYGRMGLLHQEKLPSDLDGMFIERLDCDMESIIRNDLMDGDT
LDFNFDNVLPNQSFPHSVKTTTHSWVSG (SEQ ID NO: 3)

AKT (NP 005154 gi:4885061) 480 amino acids

See, e.g. Staal, S.P., Proc. Natl. Acad. Sci. U.S.A. 84 (14), 5034-5037 (1987)

MSDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYKERPQDVDQREAPLNNFSV
AQCQLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEEREEWTTAIQTVADGLKKQE
EEEMDFRSGSPSDNSGAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKA
TGRYYAMKILKKEVIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVME
YANGGELFFHLSRERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKD
GHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEM
MCGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDA
KEIMQHRFFAGIVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDD
SMECVDSERRPHFPQFS**Y**SASSTA (SEQ ID NO: 4)

PTEN (NP 000305, gi:4506249) 403 amino acids

See, e.g. Li et al., Science 275 (5308), 1943-1947 (1997)

MTAIIKEIVSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFL
DSKHKNHYKIYNLCAERHYDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSE
DDNHVAAIHCKAGKGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPSQ
RRYVYYYSYLLKNHLDYRPVALLFHKMMFETIPMFSGGTCNPQFVVCQLKVKIYSSN
SGPTRREDKFMYFEFPQPLPVCGDIKVEFFHKQNKMLKKDKMFHFWVNTFFIPGPEE
TSEKVENGSLCDQEIDSICSIERADNDKEYLVLTLTKNDLDKANKDKANRYFSPNFK
VKLYFTKTVEEPSNPEASSSTSVTPDVSDNEPDHYRYSDTTDSDPENEPFDEDQHTQ
ITKV (SEQ ID NO: 5)

FKHRL1 (O43524, gi:8134467) 673 amino acids
See, e.g. Hillion et al., Blood 90 (9), 3714-3719 (1997).

5 MAEAPASPAPLSPLEVELDPEFEPQSRPRSCTWPLQRPELQASPAKPSGETAADSMI
PEEEDDEDDEDGGGRAGSAMAIGGGGGSGTLGSGLLLEDSARVLAPGGQDPGSGPAT
AAGGLSGGTQALLQPQQPLPPPQPGAAGGSGQPRKCSSRRNAWGNLSYADLITRAIE
SSPDKRLTLSQIYEWMVRCVPYFKDKGDSNSSAGWKNSIRHNLSLHSRFMRVQNEGT
GKSSWWIINPDGGKSGKAPRRRAVSMDNSNKYTKSRGRAAKKKAALQTAPESADDSP
10 SQLSKWPGSPTSRSSDELDAWTDFRSRTNSNASTVSGRLSPIMASTELDEVQDDDAP
LSPMLYSSSASLSPSVSKPCTVELPRLTDMAGTMNLNDGLTENLMDDLLDNITLPPS
QPSPTGGLMQRSSSFPYTTKGSGLGSPTSSFNSTVFGPSSLNSLRQSPMQTIQENKP
ATFSSMSHYGNQTLQDLLTSDSLSHSDVMMTQSDPLMSQASTAVSAQNSRRNVMLRN
DPMMSFAAQPNQGSLVNQNLLHHQHQTQGALGGSRALSNSVSNMGLSESSSLGSAKH
15 QQQSPVSQSMQTLSDSLSGSSLYSTSANLPVMGHEKFPSDLDLDMFNGSLECDMESI
IRSELMDADGLDFNFDSLISTQNVVGLNVGNFTGAKQASSQSWVPG (SEQ ID NO: 6)

EGFR (NP 005219, gi:29725609) 1210 amino acids
20 See, e.g. Tam et al., Nature 309 (5967), 418-425 (1984)

MRPSGTAGAALLALLAALCPASRALEEKKVCQGTSNKLTQLGTFEDHFLSLQRMFNN
CEVVLGNLEITYVQRNYDLSFLKTIQEVAGYVLIALNTVERIPLENLQIIRGNMYYE
NSYALAVLSNYDANKTGLKELPMRNLQEILHGAVRFSNNPALCNVESIQWRDIVSSD
25 FLSNMSMDFQNHLGSCQKCDPSCPNGSCWGAGEENCQKLTKIICAQQCSGRCRGKSP
SDCCHNQCAAGCTGPRESDCLVCRKFRDEATCKDTCPPLMLYNPTTYQMDVNPEGKY
SFGATCVKKCPRNYVVTDHGSCVRACGADSYEMEEDGVRKCKKCEGPCRKVCNGIGI
GEFKDSLSINATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQELDILKTVK
EITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLNITSLGLRSLKEI
30 SDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSCKATGQVCHALCSPE
GCWGPEPRDCVSCRNVSRGRECVDKCNLLEGEPREFVENSECIQCHPECLPQAMNIT
CTGRGPDNCIQCAHYIDGPHCVKTCPAGVMGENNTLVWKYADAGHVCHLCHPNCTYG
CTGPGLEGCPTNGPKIPSIATGMVGALLLLLVVALGIGLFMRRRHIVRKRTLRRLLQ
ERELVEPLTPSGEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIP
35 VAIKELREATSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMPFGCL
LDYVREHKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQHVKITD
FGLAKLLGAEEKEYHAEGGKVPIKWMALESILHRIYTHQSDVWSYGVTVWELMTFGS
KPYDGIPASEISSILEKGERLPQPPICTIDVYMIMVKCWMIDADSRPKFRELIIEFS
KMARDPQRYLVIQGDERMHLPSPTDSNFYRALMDEEDMDDVVDADEYLIPQQGFFSS
40 PSTSRTPLLSSLSATSNNSTVACIDRNGLQSCPIKEDSFLQRYSSDPTGALTEDSID
DTFLPVPEYINQSVPKRPAGSVQNPVYHNQPLNPAPSRDPHYQDPHSTAVGNPEYLN
TVQPTCVNSTFDSPAHWAQKGSHQISLDNPDYQQDFFPKEAKPNGIFKGSTAENAEY
LRVAPQSSEFIGA (SEQ ID NO: 7)

45 **p-ERK (XP 055766, gi:20562757) 379 amino acids**
See, e.g. Butch et al., J Biol Chem. 1996 Feb 23;271(8):4230-5.

MAAAAAQGGGGGEPRRTEGVGPGVPGEVEMVKGQPFDVGPRYTQLQYIGEGAYGMVS
SAYDHVRKTRVAIKKISPFEHQTYCQRTLREIQILLRFRHENVIGIRDILRASTLEA
MRDVYIVQDLMETDLYKLLKSQQLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSN
5 LLINTTCDLKICDFGLARIADPEHDHTGFL**TEY**VATRWYRAPEIMLNSKGYTKSIDI
WSVGCILAEMLSNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLP
SKTKVAWAKLFPKSDSKALDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDEPVA
EEPFTFAMELDDLPKERLKELIFQETARFQPGVLEAP (SEQ ID NO: 8)

10 **Ki-67 (CAA46519, gi:415819) 3256 amino acids**
See, e.g. Schluter et al., J. Cell Biol. 123 (3), 513-522 (1993)

MWPTRRLVTIKRSGVDGPHFPLSLSTCLFGRGIECDIRIQLPVVSKQHCKIEIHEQE
15 AILHNFSSTNPTQVNGSVIDEPVRLKHGDVITIIDRSFRYENESLQNGRKSTEFPRK
IREQEPARRVSRSSFSSDPDEKAQDSKAYSKITEGKVSGNPQVHIKNVKEDSTADDS
KDSVAQGTTNVHSSEHAGRNGRNAADPISGDFKEISSVKLVSRYGELKSVPTTQCLD
NSKKNESPFWKLYESVKKELDVKSQKENVLQYCRKSGLQTDYATEKESADGLQGETQ
LLVSRKSRPKSGGSGHAVAEPASPEQELDQNKGKGRDVESVQTPSKAVGASFPLYEP
20 AKMKTPVQYSQQQNSPQKHKNKDLYTTGRRESVNLGKSEGFKAGDKTLTPRKLSTRN
RTPAKVEDAADSATKPENLSSKTRGSIPTDVEVLPTETEIHNEPFLTLWLTQVERKI
QKDSLSKPEKLGTTAGQMCSGLPGLSSVDINNFGDSINESEGIPLKRRRVSFGGHLR
PELFDENLPPNTPLKRGEAPTKRKSLVMHTPPVLKKIIKEQPQPSGKQESGSEIHVE
VKAQSLVISPPAPSPRKTPVASDQRRRSCKTAPASSSKSQTEVPKRGGERVATCLQK
25 RVSISRSQHDILQMICSKRRSGASEANLIVAKSWADVVKLGAKQTQTKVIKHGPQRS
MNKRQRRPATPKKPVGEVHSQFSTGHANSPCTIIIGKAHTEKVHVPARPYRVLNNFI
SNQKMDFKEDLSGIAEMFKTPVKEQPQLTSTCHIAISNSENLLGKQFQGTDSGEEPL
LPTSESFGGNVFFSAQNAAKQPSDKCSASPPLRRQCIRENGNVAKTPRNTYKMTSLE
TKTSDTETEPSKTVSTVNRSGRSTEFRNIQKLPVESKSEETNTEIVECILKRGQKAT
30 LLQQRREGEMKEIERPFETYKENIELKENDEKMKAMKRSRTWGQKCAPMSDLTDLKS
LPDTELMKDTARGQNLLQTQDHAKAPKSEKGKITKMPCQSLQPEPINTPTHTKQQLK
ASLGKVGVKEELLAVGKFTRTSGETTHTHREPAGDGKSIRTFKESPKQILDPAARVT
GMKKWPRTPKEEAQSLEDLAGFKELFQTPGPSEESMTDEKTTKIACKSPPPESVDTP
TSTKQWPKRSLRKADVEEEFLALRKLTPSAGKAMLTPKPAGGDEKDIKAFMGTPVQK
35 LDLAGTLPGSKRQLQTPKEKAQALEDLAGFKELFQTPGHTEELVAAGKTTKIPCDSP
QSDPVDTPTSTKQRPKRSIRKADVEGELLACRNLMPSAGKAMHTPKPSVGEEKDIII
FVGTPVQKLDLTENLTGSKRRPQTPKEEAQALEDLTGFKELFQTPGHTEEAVAAGKT
TKMPCESSPPESADTPTSTRRQPKTPLEKRDVQKELSALKKLTQTSGETTHTDKVPG
GEDKSINAFRETAKQKLDPAASVTGSKRHPKTKEKAQPLEDLAGWKELFQTPVCTDK
40 PTTHEKTTKIACRSQPDPVDTPTSSKPQSKRSLRKVDVEEEFFALRKRTPSAGKAMH
TPKPAVSGEKNIYAFMGTPVQKLDLTENLTGSKRRLQTPKEKAQALEDLAGFKELFQ
TRGHTEESMTNDKTAKVACKSSQPDLDKNPASSKRRLKTSLGKVGVKEELLAVGKLT
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HRDSEDI (SEQ ID NO: 9)
25

p-H3 Histone (AAH38989, gi:25058578) 136 amino acids
See, e.g. Strausberg et al., Proc. Natl. Acad. Sci. U.S.A. 99 (26), 16899-16903 (2002)
30

MARTKQTARKSTGGKAPRKQLATKAARKSAPSTGGVKKPHRYRPGTVALREIRRYQK
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Caspase-3 (P42574, gi:1169072) 277 amino acids
See, e.g. Goldberg et al., Nat. Genet. 13 (4), 442-449 (1996)

40 MENTENSVDSKSIKNLEPKIIHGSESMDSGISLDNSYKMDYPEMGLCIIINNKNFHK
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KLEFMHILTRVNRKVATEFESFSFDATFHAKKQIPCIVSMLTKELYFYH (SEQ ID
45 NO: 11)

Table 7. Univariate analysis

	Pearson Correlation	***P***
PTEN		
p-AKT	-0.46	0.00000002
p-S6	-0.16	0.07
p-FKHR	-0.09	0.27
p-ERK	-0.027	0.76
p-AKT		
p-S6	0.21	0.01
p-FKHR	-0.09	0.31
p-ERK	-0.07	0.42
p-S6		
p-FKHR	0.04	0.59
p-ERK	0.16	0.09

Table 8: Multivariate logistic regression analysis

Outcome	Covariates	Odds Ratio	95% CI	P
PTEN				
	p-AKT	0.28[a]	(0.14-0.58)	0.0005
	p-S6	0.5	(0.30-1.04)	0.065
	p-FKHR	0.99	(0.51-2.00)	0.97
p-AKT				
	p-S6	2.17	(1.09-4.32)	0.028
	p-FKHR	0.98	(0.49-1.95)	0.95

[a] Odds of a tumor being PTEN deficient if it is p-AKT positive are 3.57 times (1/0.28) greater than if it
5 is p-AKT negative

What is claimed is:

1. A method for identifying a mammalian tumor that is likely to respond, or is responsive to an EGFR polypeptide (SEQ ID NO: 7) inhibitor or an mTOR polypeptide (SEQ ID NO: 2) inhibitor, the method comprising examining a sample obtained from the tumor for:

(a) the expression of PTEN polypeptide (SEQ ID NO: 5);

and the presence of at least one of,

(b) phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1);

(c) EGFR polypeptide (SEQ ID NO: 7)

(d) phosphorylated AKT polypeptide (SEQ ID NO: 4); and

(e) phosphorylated ERK polypeptide (SEQ ID NO: 8)

wherein decreased expression of PTEN polypeptide together with decreased phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the tumor as likely to respond or responsive to an mTOR inhibitor, and

wherein decreased expression an of PTEN together with normal phosphorylation of S6 ribosomal polypeptide in the sample, as compared to a control, identifies the tumor as not likely to respond or unresponsive to an mTOR inhibitor, and

wherein normal or increased expression of PTEN and increased expression and/or activity of EGFR together with increased phosphorylation of AKT and/or phosphorylation of ERK identifies the tumor as not likely to respond and/or unresponsive to an EGFR inhibitor.

2. The method of claim 1, wherein the mammalian tumor is a glioma or a cancer of the prostate, bile duct, bladder, breast, colon, endometrium, blood, liver, lung, skin, ovary, pancreas or thyroid.

3. The method of claim 1, wherein the phosphorylation of S6 ribosomal polypeptide is determined subsequent to contacting the tumor or sample with an mTOR inhibitor and/or the phosphorylation of AKT and/or ERK is determined subsequent to contacting the tumor or sample with an EGFR inhibitor.

5

4. The method of claim 1, wherein the mTOR inhibitor is rapamycin, SDZ-RAD, CCI-779, RAD 001, or AP23573.

5. The method of claim 1, wherein the EGFR inhibitor is ZD-1839, OSI-774, PD-
10 153053, PD-168393, IMC-C225 or CI-1033.

6. The method of claim 1, wherein the expression of one or more of (a)-(e) is examined using an antibody.

15 7. The method of claim 6, wherein the presence of phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1) is examined using an antibody that binds an epitope comprising a phosphorylated serine residue at position 235 in SEQ ID NO: 1.

8. The method of claim 6, wherein the presence of EGFR and PTEN are examined
20 using an EGFR-specific antibody and PTEN-specific antibody, respectively.

9. The method of claim 6, wherein the presence of phosphorylated AKT (SEQ ID NO: 4) is examined using an antibody that binds an epitope comprising a phosphorylated serine residue at position 473 in SEQ ID NO: 4.

25

10. The method of claim 6, wherein the presence of phosphorylated ERK is examined using an antibody that binds an epitope comprising a phosphorylated threonine residue at position 202 or a phosphorylated tyrosine residue at position 204 in SEQ ID NO: 8.

30

11. The method of claim 2, wherein the tumor is a glioblastoma multiforme tumor.

12. The method of claim 2, wherein the tumor is a prostate cancer.

5 13. The method of claim 1, wherein the sample is a paraffin embedded biopsy sample.

14. A method for identifying a mammalian tumor cell that does not express a PTEN polypeptide (SEQ ID NO: 5) and which is likely to respond or is responsive to an
10 inhibitor of mTOR polypeptide (SEQ ID NO: 2) activity, the method comprising examining a sample obtained from the tumor for the presence of phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1) after contacting the tumor or the sample with the inhibitor,

wherein, an observable decrease in phosphorylated S6 ribosomal polypeptide in
15 the sample, as compared to a control that is not contacted with the inhibitor identifies the tumor cells as likely to respond or responsive to the inhibitor, and

wherein no observable decrease in phosphorylated S6 ribosomal polypeptide in the sample, as compared to a control identifies the tumor cell as not likely to respond or unresponsive to the inhibitor.

20

15. The method of claim 14, wherein the tumor is glioma.

16. The method of claim 15, wherein the glioma is identified as tumor that does not express a PTEN polypeptide (SEQ ID NO: 5) by using an antibody that binds the
25 PTEN polypeptide (SEQ ID NO: 5).

17. The method of claim 14, wherein the tumor is a prostate cancer.

18. A method for identifying a mammalian prostate tumor that expresses a PTEN polypeptide (SEQ ID NO: 5) and which is not likely to respond or is nonresponsive to

an inhibitor of EGFR polypeptide (SEQ ID NO: 7) activity, the method comprising examining a sample obtained from the tumor for the presence of EGFR (SEQ ID NO: 7) and the presence of a phosphorylated AKT polypeptide (SEQ ID NO: 4) or the presence of a phosphorylated ERK polypeptide (SEQ ID NO: 8), after contacting the
5 tumor or the sample with the inhibitor,

wherein an increase in the levels of the EGFR polypeptide and the levels of phosphorylated AKT polypeptide or phosphorylated ERK polypeptide identifies the prostate tumor as not likely to respond or nonresponsive to the inhibitor.

10 19. The method of claim 18, wherein the a sample obtained from the tumor is examined for the presence of a phosphorylated AKT polypeptide (SEQ ID NO: 4) and the presence of a phosphorylated ERK polypeptide (SEQ ID NO: 8).

20. The method of claim 18, wherein the tumor is obtained via biopsy.

15

21. The method of claim 18, wherein the tumor is identified as a tumor that expresses a PTEN polypeptide (SEQ ID NO: 5) using an antibody that binds the PTEN polypeptide (SEQ ID NO: 5).

20 22. A kit for characterizing a mammalian tumor or cell, the kit comprising:

(a) an antibody that binds PTEN (SEQ ID NO: 5);

and one or more of the following:

(b) an antibody that binds phosphorylated S6 ribosomal polypeptide (SEQ ID NO: 1);

25 (c) an antibody that binds EFGR (SEQ ID NO: 7);

(d) an antibody that binds phosphorylated AKT (SEQ ID NO: 4); and

(e) an antibody that binds phosphorylated ERK (SEQ ID NO: 8).

23. The kit of claim 22, wherein the kit comprises a plurality of antibodies selected
30 from the group consisting of (b)-(e).

24. The kit of claim 22, wherein:

the antibody of (b) is specific for S6 ribosomal polypeptide (SEQ ID NO: 1) having a phosphorylated serine residue at position 235 in SEQ ID NO: 1;

5 the antibody of (d) is specific for AKT (SEQ ID NO: 4) having a phosphorylated serine residue at position 473 in SEQ ID NO: 4; and

the antibody of (e) is specific for ERK having a phosphorylated threonine residue at position 202 and tyrosine 204 in SEQ ID NO: 8.

10 25. The kit of claim 22, wherein the kit further includes an antibody that binds Ki-67 polypeptide (SEQ ID NO: 9), p-H3 histone polypeptide (SEQ ID NO: 10) or caspase-3 polypeptide (SEQ ID NO: 11).

26. The kit of claim 22, wherein the kit further includes; and at least one secondary 15 antibody that binds to an antibody (a)-(e).

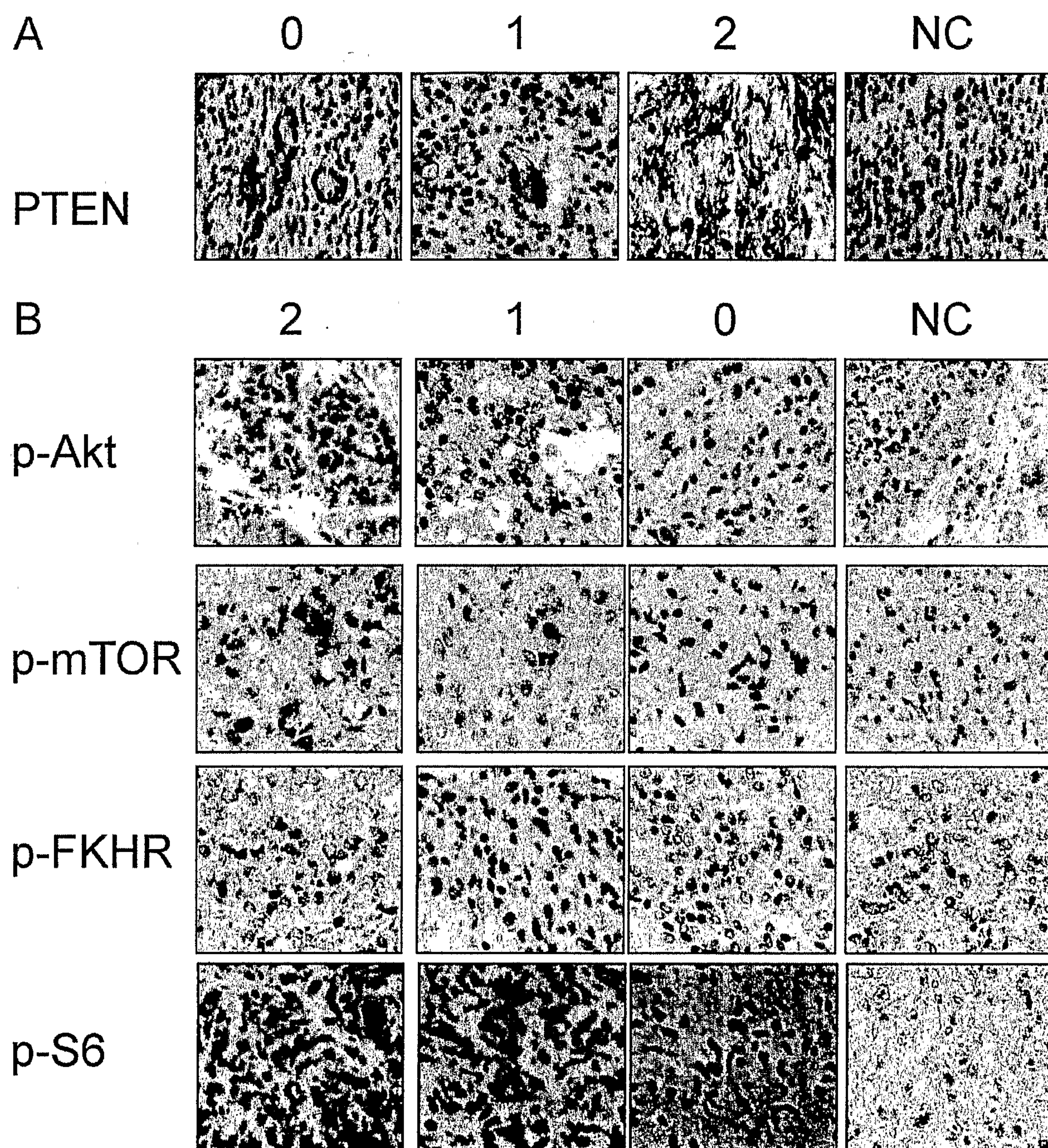


FIGURE 1

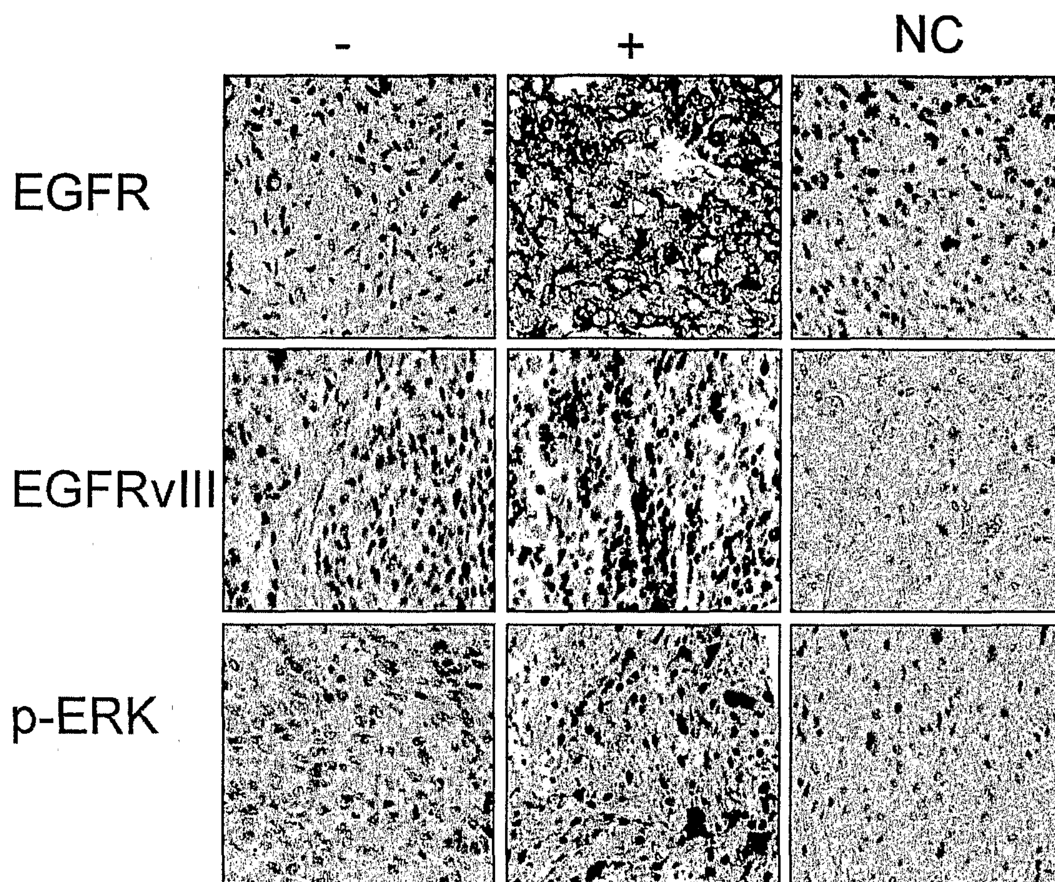


FIGURE 2

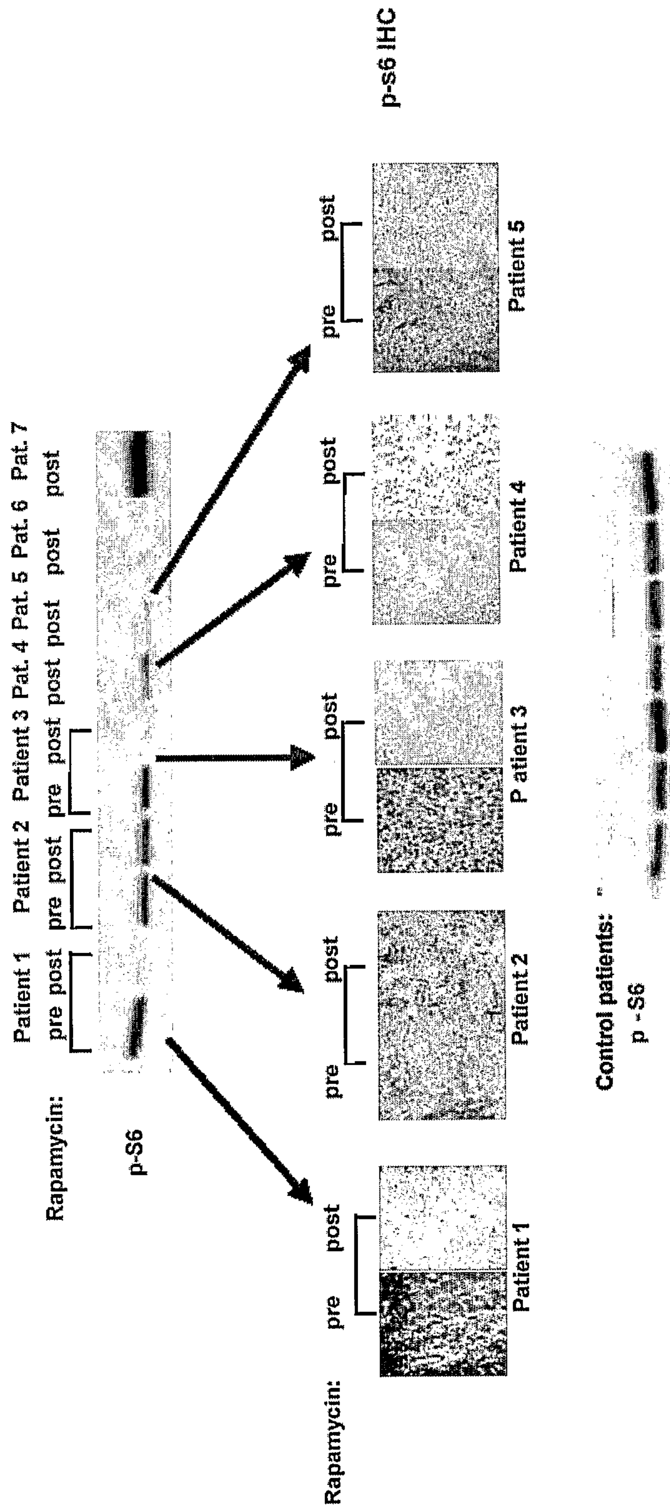


FIG. 3A

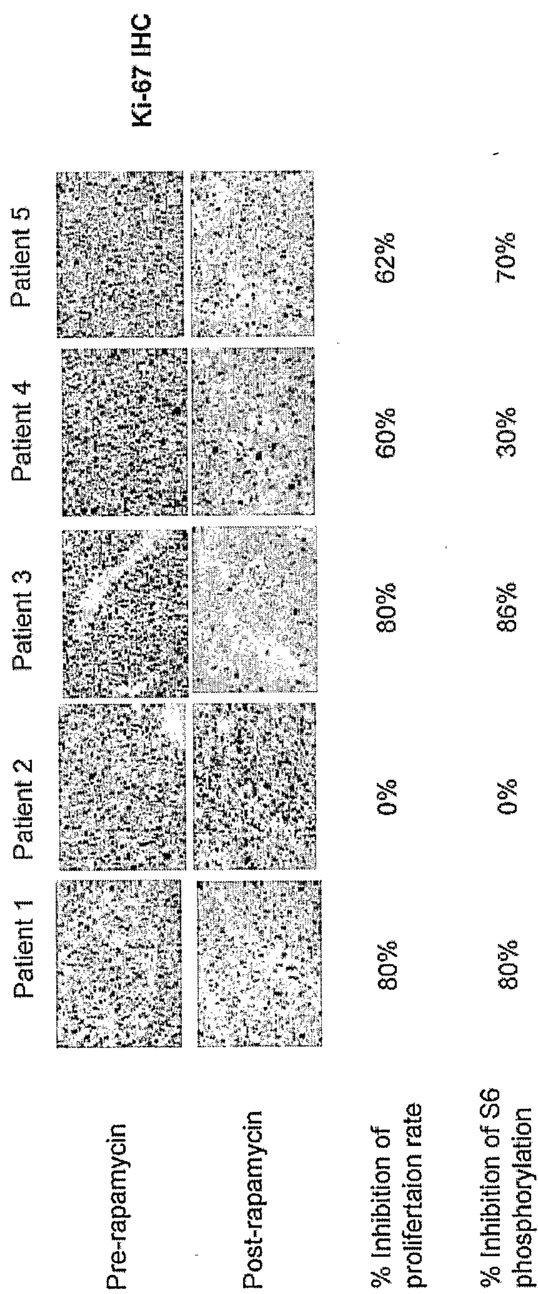


FIG. 3B

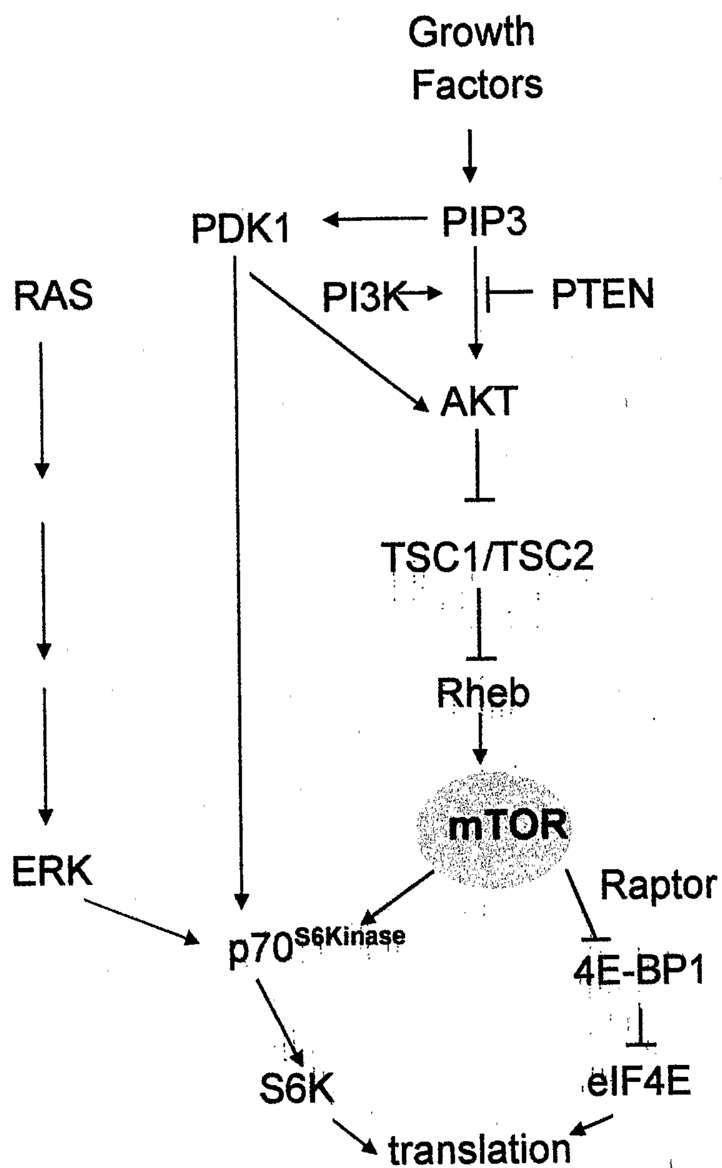
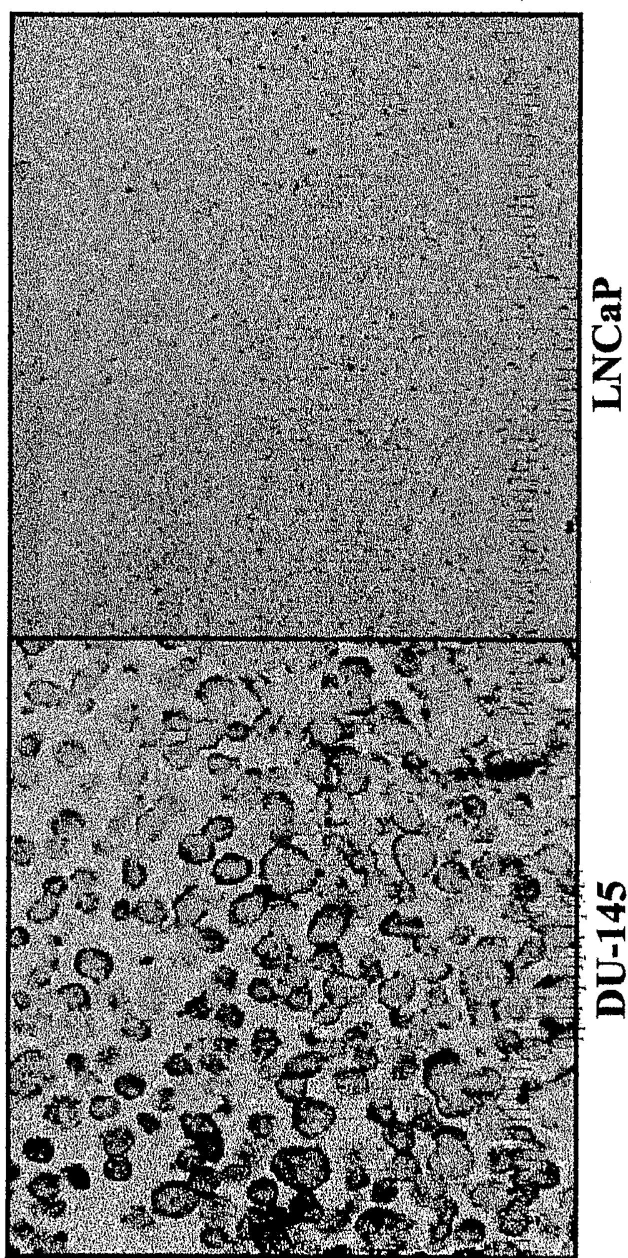


FIGURE 4



PTEN

FIGURE 5A

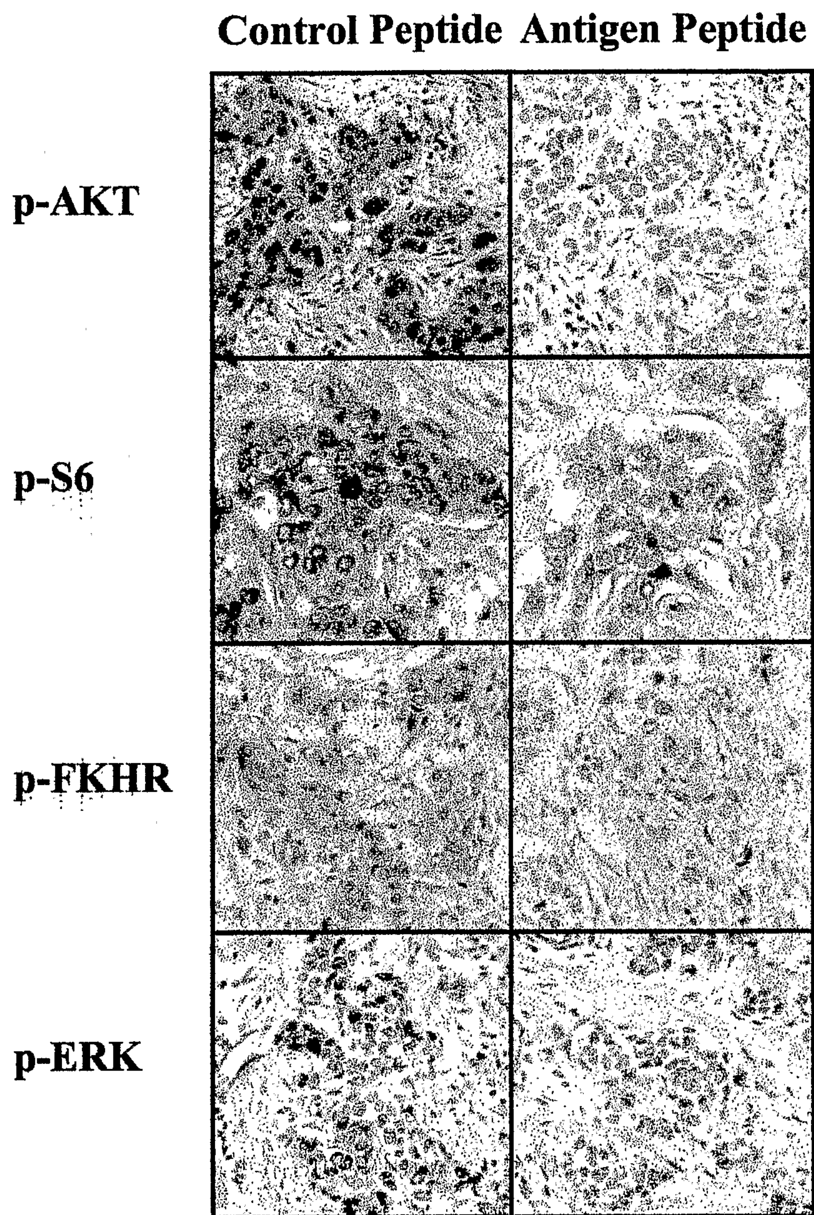


FIGURE 5B

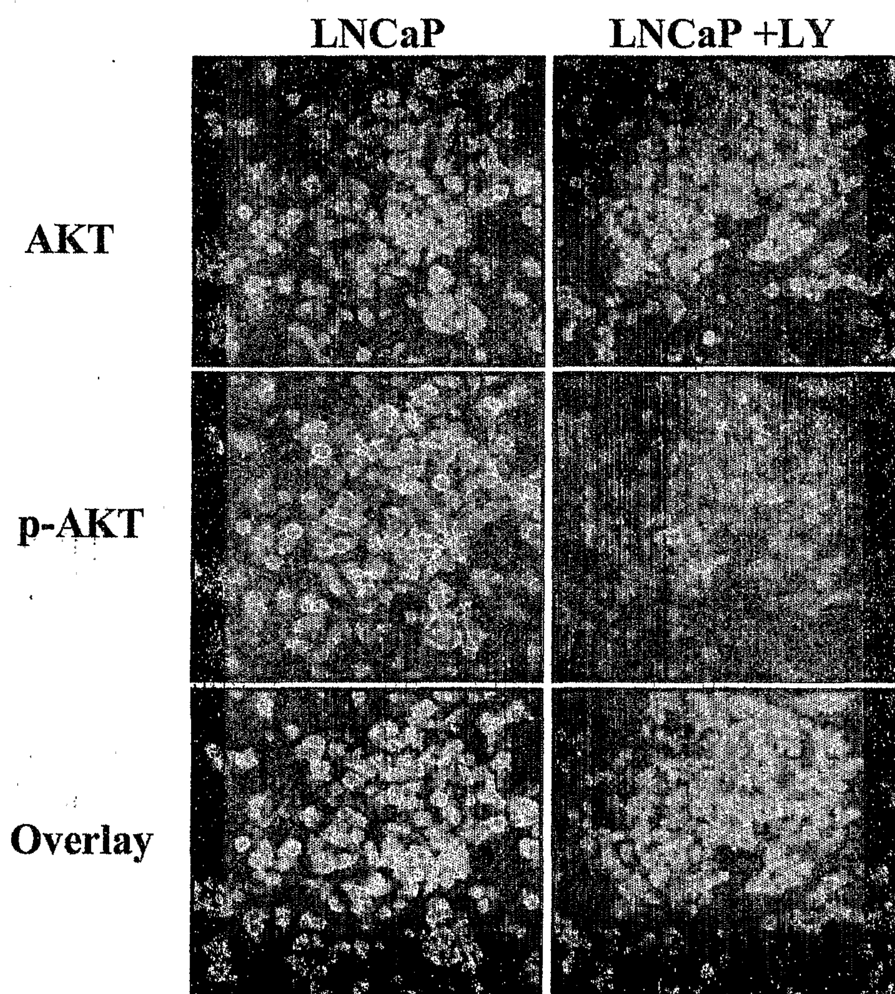


FIGURE 5C

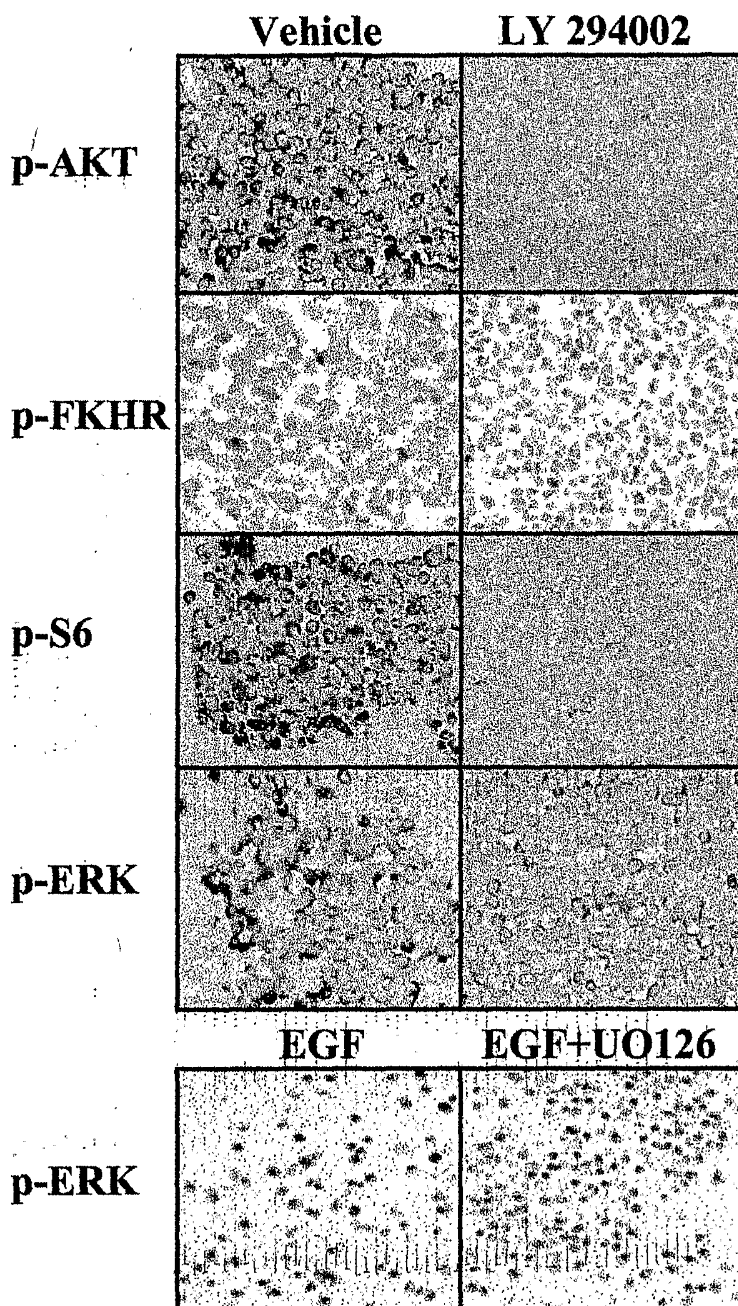


FIGURE 5D

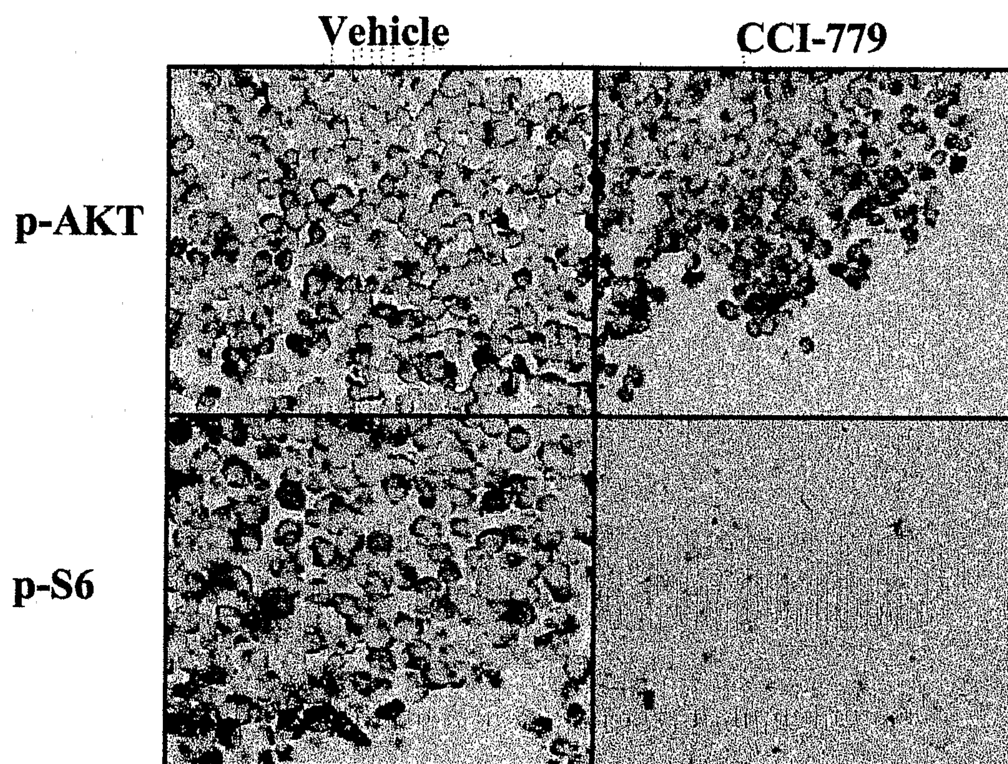


FIGURE 5E

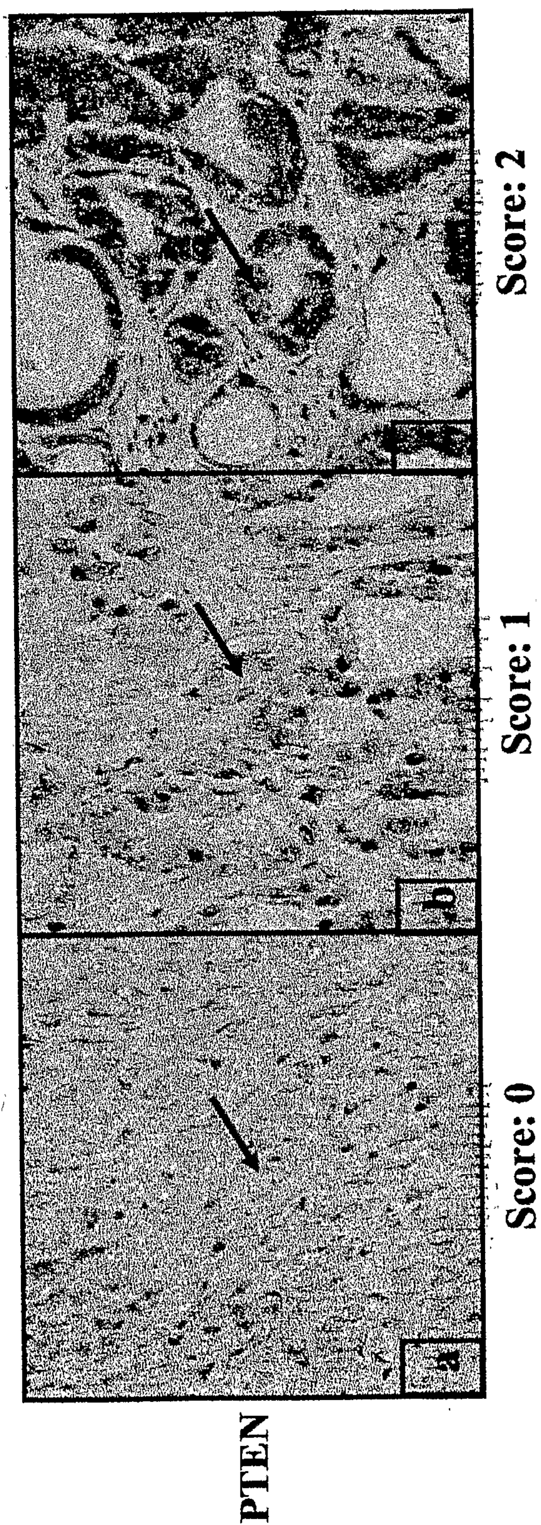


FIGURE 6A

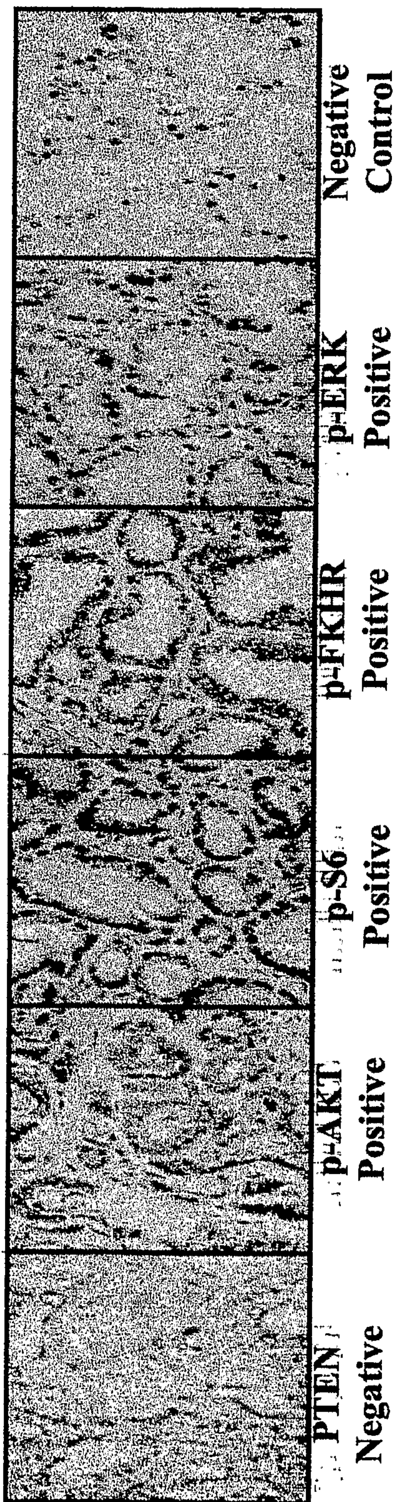


FIGURE 6B

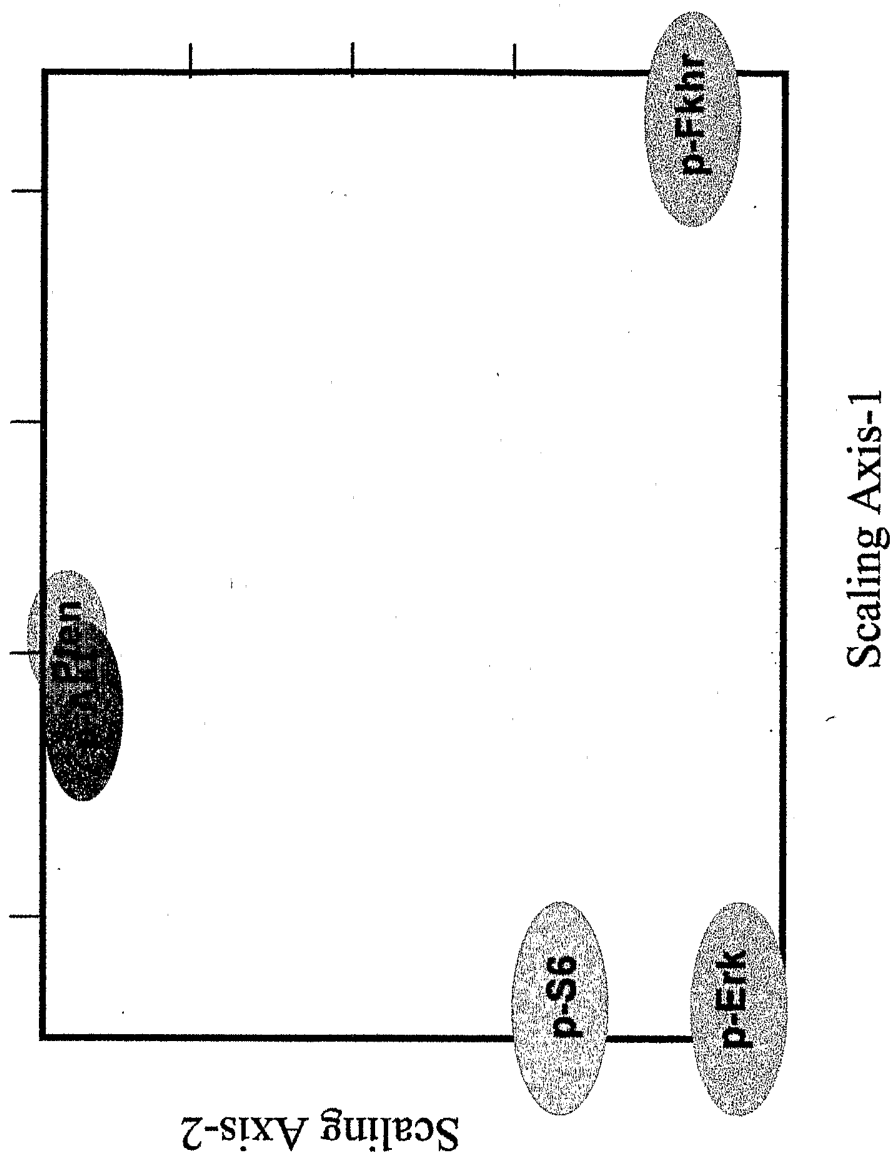


FIGURE 7

SEQUENCE LISTING

<110> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA
CELL SIGNALING TECHNOLOGY, INC.
CHARLES L. SAWYERS
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KATHERINE CROSBY
GEORGE V. THOMAS

<120> METHODS AND MATERIALS FOR EXAMINING THE PI3K/AKT
PATHWAY IN GLIOMAS AND PROSTATE CANCERS

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Gln His Arg Met Leu Arg Ser Gly Gln Gly Asp Ala Leu Ala Ser Gly
        1235                1240                1245
Pro Val Glu Thr Gly Pro Met Lys Lys Leu His Val Ser Thr Ile Asn
    1250                1255                1260
Leu Gln Lys Ala Trp Gly Ala Ala Arg Arg Val Ser Lys Asp Asp Trp
1265                1270                1275                1280
Leu Glu Trp Leu Arg Arg Leu Ser Leu Glu Leu Leu Lys Asp Ser Ser
                1285                1290                1295
Ser Pro Ser Leu Arg Ser Cys Trp Ala Leu Ala Gln Ala Tyr Asn Pro
            1300                1305                1310
Met Ala Arg Asp Leu Phe Asn Ala Ala Phe Val Ser Cys Trp Ser Glu
        1315                1320                1325
Leu Asn Glu Asp Gln Gln Asp Glu Leu Ile Arg Ser Ile Glu Leu Ala
    1330                1335                1340
Leu Thr Ser Gln Asp Ile Ala Glu Val Thr Gln Thr Leu Leu Asn Leu
1345                1350                1355                1360
```

```
Ala Glu Phe Met Glu His Ser Asp Lys Gly Pro Leu Pro Leu Arg Asp
                1365                1370                1375
Asp Asn Gly Ile Val Leu Leu Gly Glu Arg Ala Ala Lys Cys Arg Ala
            1380                1385                1390
Tyr Ala Lys Ala Leu His Tyr Lys Glu Leu Glu Phe Gln Lys Gly Pro
        1395                1400                1405
Thr Pro Ala Ile Leu Glu Ser Leu Ile Ser Ile Asn Asn Lys Leu Gln
    1410                1415                1420
Gln Pro Glu Ala Ala Ala Gly Val Leu Glu Tyr Ala Met Lys His Phe
1425                1430                1435                1440
Gly Glu Leu Glu Ile Gln Ala Thr Trp Tyr Glu Lys Leu His Glu Trp
                1445                1450                1455
Glu Asp Ala Leu Val Ala Tyr Asp Lys Lys Met Asp Thr Asn Lys Asp
            1460                1465                1470
Asp Pro Glu Leu Met Leu Gly Arg Met Arg Cys Leu Glu Ala Leu Gly
        1475                1480                1485
Glu Trp Gly Gln Leu His Gln Gln Cys Cys Glu Lys Trp Thr Leu Val
    1490                1495                1500
Asn Asp Glu Thr Gln Ala Lys Met Ala Arg Met Ala Ala Ala Ala Ala
1505                1510                1515                1520
Trp Gly Leu Gly Gln Trp Asp Ser Met Glu Glu Tyr Thr Cys Met Ile
                1525                1530                1535
Pro Arg Asp Thr His Asp Gly Ala Phe Tyr Arg Ala Val Leu Ala Leu
            1540                1545                1550
His Gln Asp Leu Phe Ser Leu Ala Gln Gln Cys Ile Asp Lys Ala Arg
        1555                1560                1565
Asp Leu Leu Asp Ala Glu Leu Thr Ala Met Ala Gly Glu Ser Tyr Ser
    1570                1575                1580
Arg Ala Tyr Gly Ala Met Val Ser Cys His Met Leu Ser Glu Leu Glu
1585                1590                1595                1600
Glu Val Ile Gln Tyr Lys Leu Val Pro Glu Arg Arg Glu Ile Ile Arg
                1605                1610                1615
Gln Ile Trp Trp Glu Arg Leu Gln Gly Cys Gln Arg Ile Val Glu Asp
            1620                1625                1630
Trp Gln Lys Ile Leu Met Val Arg Ser Leu Val Val Ser Pro His Glu
        1635                1640                1645
Asp Met Arg Thr Trp Leu Lys Tyr Ala Ser Leu Cys Gly Lys Ser Gly
    1650                1655                1660
Arg Leu Ala Leu Ala His Lys Thr Leu Val Leu Leu Leu Gly Val Asp
1665                1670                1675                1680
Pro Ser Arg Gln Leu Asp His Pro Leu Pro Thr Val His Pro Gln Val
                1685                1690                1695
Thr Tyr Ala Tyr Met Lys Asn Met Trp Lys Ser Ala Arg Lys Ile Asp
            1700                1705                1710
Ala Phe Gln His Met Gln His Phe Val Gln Thr Met Gln Gln Gln Ala
        1715                1720                1725
Gln His Ala Ile Ala Thr Glu Asp Gln Gln His Lys Gln Glu Leu His
    1730                1735                1740
Lys Leu Met Ala Arg Cys Phe Leu Lys Leu Gly Glu Trp Gln Leu Asn
1745                1750                1755                1760
Leu Gln Gly Ile Asn Glu Ser Thr Ile Pro Lys Val Leu Gln Tyr Tyr
                1765                1770                1775
Ser Ala Ala Thr Glu His Asp Arg Ser Trp Tyr Lys Ala Trp His Ala
            1780                1785                1790
Trp Ala Val Met Asn Phe Glu Ala Val Leu His Tyr Lys His Gln Asn
        1795                1800                1805
Gln Ala Arg Asp Glu Lys Lys Lys Leu Arg His Ala Ser Gly Ala Asn
    1810                1815                1820
Ile Thr Asn Ala Thr Thr Ala Ala Thr Thr Ala Ala Thr Ala Thr Thr
```

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1825                1830                1835                1840
Thr Ala Ser Thr Glu Gly Ser Asn Ser Glu Ser Glu Ala Glu Ser Thr
                1845                1850                1855
Glu Asn Ser Pro Thr Pro Ser Pro Leu Gln Lys Lys Val Thr Glu Asp
            1860                1865                1870
Leu Ser Lys Thr Leu Leu Met Tyr Thr Val Pro Ala Val Gln Gly Phe
        1875                1880                1885
Phe Arg Ser Ile Ser Leu Ser Arg Gly Asn Asn Leu Gln Asp Thr Leu
    1890                1895                1900
Arg Val Leu Thr Leu Trp Phe Asp Tyr Gly His Trp Pro Asp Val Asn
1905                1910                1915                1920
Glu Ala Leu Val Glu Gly Val Lys Ala Ile Gln Ile Asp Thr Trp Leu
                1925                1930                1935
Gln Val Ile Pro Gln Leu Ile Ala Arg Ile Asp Thr Pro Arg Pro Leu
            1940                1945                1950
Val Gly Arg Leu Ile His Gln Leu Leu Thr Asp Ile Gly Arg Tyr His
        1955                1960                1965
Pro Gln Ala Leu Ile Tyr Pro Leu Thr Val Ala Ser Lys Ser Thr Thr
    1970                1975                1980
Thr Ala Arg His Asn Ala Ala Asn Lys Ile Leu Lys Asn Met Cys Glu
1985                1990                1995                2000
His Ser Asn Thr Leu Val Gln Gln Ala Met Met Val Ser Glu Glu Leu
                2005                2010                2015
Ile Arg Val Ala Ile Leu Trp His Glu Met Trp His Glu Gly Leu Glu
            2020                2025                2030
Glu Ala Ser Arg Leu Tyr Phe Gly Glu Arg Asn Val Lys Gly Met Phe
        2035                2040                2045
Glu Val Leu Glu Pro Leu His Ala Met Met Glu Arg Gly Pro Gln Thr
    2050                2055                2060
Leu Lys Glu Thr Ser Phe Asn Gln Ala Tyr Gly Arg Asp Leu Met Glu
2065                2070                2075                2080
Ala Gln Glu Trp Cys Arg Lys Tyr Met Lys Ser Gly Asn Val Lys Asp
                2085                2090                2095
Leu Thr Gln Ala Trp Asp Leu Tyr Tyr His Val Phe Arg Arg Ile Ser
            2100                2105                2110
Lys Gln Leu Pro Gln Leu Thr Ser Leu Glu Leu Gln Tyr Val Ser Pro
        2115                2120                2125
Lys Leu Leu Met Cys Arg Asp Leu Glu Leu Ala Val Pro Gly Thr Tyr
    2130                2135                2140
Asp Pro Asn Gln Pro Ile Ile Arg Ile Gln Ser Ile Ala Pro Ser Leu
2145                2150                2155                2160
Gln Val Ile Thr Ser Lys Gln Arg Pro Arg Lys Leu Thr Leu Met Gly
                2165                2170                2175
Ser Asn Gly His Glu Phe Val Phe Leu Leu Lys Gly His Glu Asp Leu
            2180                2185                2190
Arg Gln Asp Glu Arg Val Met Gln Leu Phe Gly Leu Val Asn Thr Leu
        2195                2200                2205
Leu Ala Asn Asp Pro Thr Ser Leu Arg Lys Asn Leu Ser Ile Gln Arg
    2210                2215                2220
Tyr Ala Val Ile Pro Leu Ser Thr Asn Ser Gly Leu Ile Gly Trp Val
2225                2230                2235                2240
Pro His Cys Asp Thr Leu His Ala Leu Ile Arg Asp Tyr Arg Glu Lys
                2245                2250                2255
Lys Lys Ile Leu Leu Asn Ile Glu His Arg Ile Met Leu Arg Met Ala
            2260                2265                2270
Pro Asp Tyr Asp His Leu Thr Leu Met Gln Lys Val Glu Val Phe Glu
        2275                2280                2285
His Ala Val Asn Asn Thr Ala Gly Asp Asp Leu Ala Lys Leu Leu Trp
    2290                2295                2300
```

```
Leu Lys Ser Pro Ser Ser Glu Val Trp Phe Asp Arg Arg Thr Asn Tyr
2305                2310                2315                2320
Thr Arg Ser Leu Ala Val Met Ser Met Val Gly Tyr Ile Leu Gly Leu
                2325                2330                2335
Gly Asp Arg His Pro Ser Asn Leu Met Leu Asp Arg Leu Ser Gly Lys
            2340                2345                2350
Ile Leu His Ile Asp Phe Gly Asp Cys Phe Glu Val Ala Met Thr Arg
        2355                2360                2365
Glu Lys Phe Pro Glu Lys Ile Pro Phe Arg Leu Thr Arg Met Leu Thr
    2370                2375                2380
Asn Ala Met Glu Val Thr Gly Leu Asp Gly Asn Tyr Arg Ile Thr Cys
2385                2390                2395                2400
His Thr Val Met Glu Val Leu Arg Glu His Lys Asp Ser Val Met Ala
                2405                2410                2415
Val Leu Glu Ala Phe Val Tyr Asp Pro Leu Leu Asn Trp Arg Leu Met
            2420                2425                2430
Asp Thr Asn Thr Lys Gly Asn Lys Arg Ser Arg Thr Arg Thr Asp Ser
        2435                2440                2445
Tyr Ser Ala Gly Gln Ser Val Glu Ile Leu Asp Gly Val Glu Leu Gly
    2450                2455                2460
Glu Pro Ala His Lys Lys Thr Gly Thr Thr Val Pro Glu Ser Ile His
2465                2470                2475                2480
Ser Phe Ile Gly Asp Gly Leu Val Lys Pro Glu Ala Leu Asn Lys Lys
                2485                2490                2495
Ala Ile Gln Ile Ile Asn Arg Val Arg Asp Lys Leu Thr Gly Arg Asp
            2500                2505                2510
Phe Ser His Asp Asp Thr Leu Asp Val Pro Thr Gln Val Glu Leu Leu
        2515                2520                2525
Ile Lys Gln Ala Thr Ser His Glu Asn Leu Cys Gln Cys Tyr Ile Gly
    2530                2535                2540
Trp Cys Pro Phe Trp
2545

<210> 3
<211> 655
<212> PRT
<213> Homo Sapiens

<400> 3
Met Ala Glu Ala Pro Gln Val Val Glu Ile Asp Pro Asp Phe Glu Pro
 1               5                  10                  15
Leu Pro Arg Pro Arg Ser Cys Thr Trp Pro Leu Pro Arg Pro Glu Phe
            20                  25                  30
Ser Gln Ser Asn Ser Ala Thr Ser Ser Pro Ala Pro Ser Gly Ser Ala
        35                  40                  45
Ala Ala Asn Pro Asp Ala Ala Ala Gly Leu Pro Ser Ala Ser Ala Ala
    50                  55                  60
Ala Val Ser Ala Asp Phe Met Ser Asn Leu Ser Leu Leu Glu Glu Ser
65                  70                  75                  80
Glu Asp Phe Pro Gln Ala Pro Gly Ser Val Ala Ala Ala Val Ala Ala
                85                  90                  95
Ala Ala Ala Ala Ala Ala Thr Gly Gly Leu Cys Gly Asp Phe Gln Gly
            100                 105                 110
Pro Glu Ala Gly Cys Leu His Pro Ala Pro Pro Gln Pro Pro Pro Pro
        115                 120                 125
Gly Pro Leu Ser Gln His Pro Pro Val Pro Pro Ala Ala Ala Gly Pro
    130                 135                 140
Leu Ala Gly Gln Pro Arg Lys Ser Ser Ser Ser Arg Arg Asn Ala Trp
145                 150                 155                 160
```

```
Gly Asn Leu Ser Tyr Ala Asp Leu Ile Thr Lys Ala Ile Glu Ser Ser
                165                 170                 175
Ala Glu Lys Arg Leu Thr Leu Ser Gln Ile Tyr Glu Trp Met Val Lys
            180                 185                 190
Ser Val Pro Tyr Phe Lys Asp Lys Gly Asp Ser Asn Ser Ser Ala Gly
        195                 200                 205
Trp Lys Asn Ser Ile Arg His Asn Leu Ser Leu His Ser Lys Phe Ile
    210                 215                 220
Arg Val Gln Asn Glu Gly Thr Gly Lys Ser Ser Trp Trp Met Leu Asn
225                 230                 235                 240
Pro Glu Gly Gly Lys Ser Gly Lys Ser Pro Arg Arg Arg Ala Ala Ser
                245                 250                 255
Met Asp Asn Asn Ser Lys Phe Ala Lys Ser Arg Ser Arg Ala Ala Lys
            260                 265                 270
Lys Lys Ala Ser Leu Gln Ser Gly Gln Glu Gly Ala Gly Asp Ser Pro
        275                 280                 285
Gly Ser Gln Phe Ser Lys Trp Pro Ala Ser Pro Gly Ser His Ser Asn
    290                 295                 300
Asp Asp Phe Asp Asn Trp Ser Thr Phe Arg Pro Arg Thr Ser Ser Asn
305                 310                 315                 320
Ala Ser Thr Ile Ser Gly Arg Leu Ser Pro Ile Met Thr Glu Gln Asp
                325                 330                 335
Asp Leu Gly Glu Gly Asp Val His Ser Met Val Tyr Pro Pro Ser Ala
            340                 345                 350
Ala Lys Met Ala Ser Thr Leu Pro Ser Leu Ser Glu Ile Ser Asn Pro
        355                 360                 365
Glu Asn Met Glu Asn Leu Leu Asp Asn Leu Asn Leu Leu Ser Ser Pro
    370                 375                 380
Thr Ser Leu Thr Val Ser Thr Gln Ser Ser Pro Gly Thr Met Met Gln
385                 390                 395                 400
Gln Thr Pro Cys Tyr Ser Phe Ala Pro Pro Asn Thr Ser Leu Asn Ser
                405                 410                 415
Pro Ser Pro Asn Tyr Gln Lys Tyr Thr Tyr Gly Gln Ser Ser Met Ser
            420                 425                 430
Pro Leu Pro Gln Met Pro Ile Gln Thr Leu Gln Asp Asn Lys Ser Ser
        435                 440                 445
Tyr Gly Gly Met Ser Gln Tyr Asn Cys Ala Pro Gly Leu Leu Lys Glu
    450                 455                 460
Leu Leu Thr Ser Asp Ser Pro Pro His Asn Asp Ile Met Thr Pro Val
465                 470                 475                 480
Asp Pro Gly Val Ala Gln Pro Asn Ser Arg Val Leu Gly Gln Asn Val
                485                 490                 495
Met Met Gly Pro Asn Ser Val Met Ser Thr Tyr Gly Ser Gln Ala Ser
            500                 505                 510
His Asn Lys Met Met Asn Pro Ser Ser His Thr His Pro Gly His Ala
        515                 520                 525
Gln Gln Thr Ser Ala Val Asn Gly Arg Pro Leu Pro His Thr Val Ser
    530                 535                 540
Thr Met Pro His Thr Ser Gly Met Asn Arg Leu Thr Gln Val Lys Thr
545                 550                 555                 560
Pro Val Gln Val Pro Leu Pro His Pro Met Gln Met Ser Ala Leu Gly
                565                 570                 575
Gly Tyr Ser Ser Val Ser Ser Cys Asn Gly Tyr Gly Arg Met Gly Leu
            580                 585                 590
Leu His Gln Glu Lys Leu Pro Ser Asp Leu Asp Gly Met Phe Ile Glu
        595                 600                 605
Arg Leu Asp Cys Asp Met Glu Ser Ile Ile Arg Asn Asp Leu Met Asp
    610                 615                 620
Gly Asp Thr Leu Asp Phe Asn Phe Asp Asn Val Leu Pro Asn Gln Ser
```

```
625                 630                 635                 640
Phe Pro His Ser Val Lys Thr Thr Thr His Ser Trp Val Ser Gly
                645                 650                 655

<210> 4
<211> 480
<212> PRT
<213> Homo Sapiens

<400> 4
Met Ser Asp Val Ala Ile Val Lys Glu Gly Trp Leu His Lys Arg Gly
 1               5                  10                  15
Glu Tyr Ile Lys Thr Trp Arg Pro Arg Tyr Phe Leu Leu Lys Asn Asp
            20                  25                  30
Gly Thr Phe Ile Gly Tyr Lys Glu Arg Pro Gln Asp Val Asp Gln Arg
        35                  40                  45
Glu Ala Pro Leu Asn Asn Phe Ser Val Ala Gln Cys Gln Leu Met Lys
    50                  55                  60
Thr Glu Arg Pro Arg Pro Asn Thr Phe Ile Ile Arg Cys Leu Gln Trp
65                  70                  75                  80
Thr Thr Val Ile Glu Arg Thr Phe His Val Glu Thr Pro Glu Glu Arg
                85                  90                  95
Glu Glu Trp Thr Thr Ala Ile Gln Thr Val Ala Asp Gly Leu Lys Lys
            100                 105                 110
Gln Glu Glu Glu Glu Met Asp Phe Arg Ser Gly Ser Pro Ser Asp Asn
        115                 120                 125
Ser Gly Ala Glu Glu Met Glu Val Ser Leu Ala Lys Pro Lys His Arg
    130                 135                 140
Val Thr Met Asn Glu Phe Glu Tyr Leu Lys Leu Leu Gly Lys Gly Thr
145                 150                 155                 160
Phe Gly Lys Val Ile Leu Val Lys Glu Lys Ala Thr Gly Arg Tyr Tyr
                165                 170                 175
Ala Met Lys Ile Leu Lys Lys Glu Val Ile Val Ala Lys Asp Glu Val
            180                 185                 190
Ala His Thr Leu Thr Glu Asn Arg Val Leu Gln Asn Ser Arg His Pro
        195                 200                 205
Phe Leu Thr Ala Leu Lys Tyr Ser Phe Gln Thr His Asp Arg Leu Cys
    210                 215                 220
Phe Val Met Glu Tyr Ala Asn Gly Gly Glu Leu Phe Phe His Leu Ser
225                 230                 235                 240
Arg Glu Arg Val Phe Ser Glu Asp Arg Ala Arg Phe Tyr Gly Ala Glu
                245                 250                 255
Ile Val Ser Ala Leu Asp Tyr Leu His Ser Glu Lys Asn Val Val Tyr
            260                 265                 270
Arg Asp Leu Lys Leu Glu Asn Leu Met Leu Asp Lys Asp Gly His Ile
        275                 280                 285
Lys Ile Thr Asp Phe Gly Leu Cys Lys Glu Gly Ile Lys Asp Gly Ala
    290                 295                 300
Thr Met Lys Thr Phe Cys Gly Thr Pro Glu Tyr Leu Ala Pro Glu Val
305                 310                 315                 320
Leu Glu Asp Asn Asp Tyr Gly Arg Ala Val Asp Trp Trp Gly Leu Gly
                325                 330                 335
Val Val Met Tyr Glu Met Met Cys Gly Arg Leu Pro Phe Tyr Asn Gln
            340                 345                 350
Asp His Glu Lys Leu Phe Glu Leu Ile Leu Met Glu Glu Ile Arg Phe
        355                 360                 365
Pro Arg Thr Leu Gly Pro Glu Ala Lys Ser Leu Leu Ser Gly Leu Leu
    370                 375                 380
Lys Lys Asp Pro Lys Gln Arg Leu Gly Gly Gly Ser Glu Asp Ala Lys
```

```
385                 390                 395                 400
Glu Ile Met Gln His Arg Phe Phe Ala Gly Ile Val Trp Gln His Val
                405                 410                 415
Tyr Glu Lys Lys Leu Ser Pro Pro Phe Lys Pro Gln Val Thr Ser Glu
            420                 425                 430
Thr Asp Thr Arg Tyr Phe Asp Glu Glu Phe Thr Ala Gln Met Ile Thr
        435                 440                 445
Ile Thr Pro Pro Asp Gln Asp Asp Ser Met Glu Cys Val Asp Ser Glu
    450                 455                 460
Arg Arg Pro His Phe Pro Gln Phe Ser Tyr Ser Ala Ser Ser Thr Ala
465                 470                 475                 480

<210> 5
<211> 403
<212> PRT
<213> Homo Sapiens

<400> 5
Met Thr Ala Ile Ile Lys Glu Ile Val Ser Arg Asn Lys Arg Arg Tyr
 1               5                  10                  15
Gln Glu Asp Gly Phe Asp Leu Asp Leu Thr Tyr Ile Tyr Pro Asn Ile
            20                  25                  30
Ile Ala Met Gly Phe Pro Ala Glu Arg Leu Glu Gly Val Tyr Arg Asn
        35                  40                  45
Asn Ile Asp Asp Val Val Arg Phe Leu Asp Ser Lys His Lys Asn His
    50                  55                  60
Tyr Lys Ile Tyr Asn Leu Cys Ala Glu Arg His Tyr Asp Thr Ala Lys
65                  70                  75                  80
Phe Asn Cys Arg Val Ala Gln Tyr Pro Phe Glu Asp His Asn Pro Pro
                85                  90                  95
Gln Leu Glu Leu Ile Lys Pro Phe Cys Glu Asp Leu Asp Gln Trp Leu
            100                 105                 110
Ser Glu Asp Asp Asn His Val Ala Ala Ile His Cys Lys Ala Gly Lys
        115                 120                 125
Gly Arg Thr Gly Val Met Ile Cys Ala Tyr Leu Leu His Arg Gly Lys
    130                 135                 140
Phe Leu Lys Ala Gln Glu Ala Leu Asp Phe Tyr Gly Glu Val Arg Thr
145                 150                 155                 160
Arg Asp Lys Lys Gly Val Thr Ile Pro Ser Gln Arg Arg Tyr Val Tyr
                165                 170                 175
Tyr Tyr Ser Tyr Leu Leu Lys Asn His Leu Asp Tyr Arg Pro Val Ala
            180                 185                 190
Leu Leu Phe His Lys Met Met Phe Glu Thr Ile Pro Met Phe Ser Gly
        195                 200                 205
Gly Thr Cys Asn Pro Gln Phe Val Val Cys Gln Leu Lys Val Lys Ile
    210                 215                 220
Tyr Ser Ser Asn Ser Gly Pro Thr Arg Arg Glu Asp Lys Phe Met Tyr
225                 230                 235                 240
Phe Glu Phe Pro Gln Pro Leu Pro Val Cys Gly Asp Ile Lys Val Glu
                245                 250                 255
Phe Phe His Lys Gln Asn Lys Met Leu Lys Lys Asp Lys Met Phe His
            260                 265                 270
Phe Trp Val Asn Thr Phe Phe Ile Pro Gly Pro Glu Glu Thr Ser Glu
        275                 280                 285
Lys Val Glu Asn Gly Ser Leu Cys Asp Gln Glu Ile Asp Ser Ile Cys
    290                 295                 300
Ser Ile Glu Arg Ala Asp Asn Asp Lys Glu Tyr Leu Val Leu Thr Leu
305                 310                 315                 320
Thr Lys Asn Asp Leu Asp Lys Ala Asn Lys Asp Lys Ala Asn Arg Tyr
```

```
                325                 330                 335
Phe Ser Pro Asn Phe Lys Val Lys Leu Tyr Phe Thr Lys Thr Val Glu
            340                 345                 350
Glu Pro Ser Asn Pro Glu Ala Ser Ser Ser Thr Ser Val Thr Pro Asp
        355                 360                 365
Val Ser Asp Asn Glu Pro Asp His Tyr Arg Tyr Ser Asp Thr Thr Asp
    370                 375                 380
Ser Asp Pro Glu Asn Glu Pro Phe Asp Glu Asp Gln His Thr Gln Ile
385                 390                 395                 400
Thr Lys Val


<210> 6
<211> 673
<212> PRT
<213> Homo Sapiens

<400> 6
Met Ala Glu Ala Pro Ala Ser Pro Ala Pro Leu Ser Pro Leu Glu Val
 1               5                  10                  15
Glu Leu Asp Pro Glu Phe Glu Pro Gln Ser Arg Pro Arg Ser Cys Thr
            20                  25                  30
Trp Pro Leu Gln Arg Pro Glu Leu Gln Ala Ser Pro Ala Lys Pro Ser
        35                  40                  45
Gly Glu Thr Ala Ala Asp Ser Met Ile Pro Glu Glu Glu Asp Asp Glu
    50                  55                  60
Asp Asp Glu Asp Gly Gly Gly Arg Ala Gly Ser Ala Met Ala Ile Gly
65                  70                  75                  80
Gly Gly Gly Gly Ser Gly Thr Leu Gly Ser Gly Leu Leu Leu Glu Asp
                85                  90                  95
Ser Ala Arg Val Leu Ala Pro Gly Gly Gln Asp Pro Gly Ser Gly Pro
            100                 105                 110
Ala Thr Ala Ala Gly Gly Leu Ser Gly Gly Thr Gln Ala Leu Leu Gln
        115                 120                 125
Pro Gln Gln Pro Leu Pro Pro Pro Gln Pro Gly Ala Ala Gly Gly Ser
    130                 135                 140
Gly Gln Pro Arg Lys Cys Ser Ser Arg Arg Asn Ala Trp Gly Asn Leu
145                 150                 155                 160
Ser Tyr Ala Asp Leu Ile Thr Arg Ala Ile Glu Ser Ser Pro Asp Lys
                165                 170                 175
Arg Leu Thr Leu Ser Gln Ile Tyr Glu Trp Met Val Arg Cys Val Pro
            180                 185                 190
Tyr Phe Lys Asp Lys Gly Asp Ser Asn Ser Ser Ala Gly Trp Lys Asn
        195                 200                 205
Ser Ile Arg His Asn Leu Ser Leu His Ser Arg Phe Met Arg Val Gln
    210                 215                 220
Asn Glu Gly Thr Gly Lys Ser Ser Trp Trp Ile Ile Asn Pro Asp Gly
225                 230                 235                 240
Gly Lys Ser Gly Lys Ala Pro Arg Arg Arg Ala Val Ser Met Asp Asn
                245                 250                 255
Ser Asn Lys Tyr Thr Lys Ser Arg Gly Arg Ala Ala Lys Lys Lys Ala
            260                 265                 270
Ala Leu Gln Thr Ala Pro Glu Ser Ala Asp Asp Ser Pro Ser Gln Leu
        275                 280                 285
Ser Lys Trp Pro Gly Ser Pro Thr Ser Arg Ser Ser Asp Glu Leu Asp
    290                 295                 300
Ala Trp Thr Asp Phe Arg Ser Arg Thr Asn Ser Asn Ala Ser Thr Val
305                 310                 315                 320
Ser Gly Arg Leu Ser Pro Ile Met Ala Ser Thr Glu Leu Asp Glu Val
```

```
                325                 330                 335
Gln Asp Asp Asp Ala Pro Leu Ser Pro Met Leu Tyr Ser Ser Ser Ala
            340                 345                 350
Ser Leu Ser Pro Ser Val Ser Lys Pro Cys Thr Val Glu Leu Pro Arg
        355                 360                 365
Leu Thr Asp Met Ala Gly Thr Met Asn Leu Asn Asp Gly Leu Thr Glu
    370                 375                 380
Asn Leu Met Asp Asp Leu Leu Asp Asn Ile Thr Leu Pro Pro Ser Gln
385                 390                 395                 400
Pro Ser Pro Thr Gly Gly Leu Met Gln Arg Ser Ser Ser Phe Pro Tyr
                405                 410                 415
Thr Thr Lys Gly Ser Gly Leu Gly Ser Pro Thr Ser Ser Phe Asn Ser
            420                 425                 430
Thr Val Phe Gly Pro Ser Ser Leu Asn Ser Leu Arg Gln Ser Pro Met
        435                 440                 445
Gln Thr Ile Gln Glu Asn Lys Pro Ala Thr Phe Ser Ser Met Ser His
    450                 455                 460
Tyr Gly Asn Gln Thr Leu Gln Asp Leu Leu Thr Ser Asp Ser Leu Ser
465                 470                 475                 480
His Ser Asp Val Met Met Thr Gln Ser Asp Pro Leu Met Ser Gln Ala
                485                 490                 495
Ser Thr Ala Val Ser Ala Gln Asn Ser Arg Arg Asn Val Met Leu Arg
            500                 505                 510
Asn Asp Pro Met Met Ser Phe Ala Ala Gln Pro Asn Gln Gly Ser Leu
        515                 520                 525
Val Asn Gln Asn Leu Leu His His Gln His Gln Thr Gln Gly Ala Leu
    530                 535                 540
Gly Gly Ser Arg Ala Leu Ser Asn Ser Val Ser Asn Met Gly Leu Ser
545                 550                 555                 560
Glu Ser Ser Ser Leu Gly Ser Ala Lys His Gln Gln Gln Ser Pro Val
                565                 570                 575
Ser Gln Ser Met Gln Thr Leu Ser Asp Ser Leu Ser Gly Ser Ser Leu
            580                 585                 590
Tyr Ser Thr Ser Ala Asn Leu Pro Val Met Gly His Glu Lys Phe Pro
        595                 600                 605
Ser Asp Leu Asp Leu Asp Met Phe Asn Gly Ser Leu Glu Cys Asp Met
    610                 615                 620
Glu Ser Ile Ile Arg Ser Glu Leu Met Asp Ala Asp Gly Leu Asp Phe
625                 630                 635                 640
Asn Phe Asp Ser Leu Ile Ser Thr Gln Asn Val Val Gly Leu Asn Val
                645                 650                 655
Gly Asn Phe Thr Gly Ala Lys Gln Ala Ser Ser Gln Ser Trp Val Pro
            660                 665                 670
Gly
```

```
<210> 7
<211> 1210
<212> PRT
<213> Homo Sapiens

<400> 7
Met Arg Pro Ser Gly Thr Ala Gly Ala Ala Leu Leu Ala Leu Leu Ala
1               5                   10                  15
Ala Leu Cys Pro Ala Ser Arg Ala Leu Glu Glu Lys Lys Val Cys Gln
            20                  25                  30
Gly Thr Ser Asn Lys Leu Thr Gln Leu Gly Thr Phe Glu Asp His Phe
        35                  40                  45
Leu Ser Leu Gln Arg Met Phe Asn Asn Cys Glu Val Val Leu Gly Asn
```

```
    50                  55                  60
Leu Glu Ile Thr Tyr Val Gln Arg Asn Tyr Asp Leu Ser Phe Leu Lys
65                  70                  75                  80
Thr Ile Gln Glu Val Ala Gly Tyr Val Leu Ile Ala Leu Asn Thr Val
                85                  90                  95
Glu Arg Ile Pro Leu Glu Asn Leu Gln Ile Ile Arg Gly Asn Met Tyr
            100                 105                 110
Tyr Glu Asn Ser Tyr Ala Leu Ala Val Leu Ser Asn Tyr Asp Ala Asn
        115                 120                 125
Lys Thr Gly Leu Lys Glu Leu Pro Met Arg Asn Leu Gln Glu Ile Leu
    130                 135                 140
His Gly Ala Val Arg Phe Ser Asn Asn Pro Ala Leu Cys Asn Val Glu
145                 150                 155                 160
Ser Ile Gln Trp Arg Asp Ile Val Ser Ser Asp Phe Leu Ser Asn Met
                165                 170                 175
Ser Met Asp Phe Gln Asn His Leu Gly Ser Cys Gln Lys Cys Asp Pro
            180                 185                 190
Ser Cys Pro Asn Gly Ser Cys Trp Gly Ala Gly Glu Glu Asn Cys Gln
        195                 200                 205
Lys Leu Thr Lys Ile Ile Cys Ala Gln Gln Cys Ser Gly Arg Cys Arg
    210                 215                 220
Gly Lys Ser Pro Ser Asp Cys Cys His Asn Gln Cys Ala Ala Gly Cys
225                 230                 235                 240
Thr Gly Pro Arg Glu Ser Asp Cys Leu Val Cys Arg Lys Phe Arg Asp
                245                 250                 255
Glu Ala Thr Cys Lys Asp Thr Cys Pro Pro Leu Met Leu Tyr Asn Pro
            260                 265                 270
Thr Thr Tyr Gln Met Asp Val Asn Pro Glu Gly Lys Tyr Ser Phe Gly
        275                 280                 285
Ala Thr Cys Val Lys Lys Cys Pro Arg Asn Tyr Val Val Thr Asp His
    290                 295                 300
Gly Ser Cys Val Arg Ala Cys Gly Ala Asp Ser Tyr Glu Met Glu Glu
305                 310                 315                 320
Asp Gly Val Arg Lys Cys Lys Lys Cys Glu Gly Pro Cys Arg Lys Val
                325                 330                 335
Cys Asn Gly Ile Gly Ile Gly Glu Phe Lys Asp Ser Leu Ser Ile Asn
            340                 345                 350
Ala Thr Asn Ile Lys His Phe Lys Asn Cys Thr Ser Ile Ser Gly Asp
        355                 360                 365
Leu His Ile Leu Pro Val Ala Phe Arg Gly Asp Ser Phe Thr His Thr
    370                 375                 380
Pro Pro Leu Asp Pro Gln Glu Leu Asp Ile Leu Lys Thr Val Lys Glu
385                 390                 395                 400
Ile Thr Gly Phe Leu Leu Ile Gln Ala Trp Pro Glu Asn Arg Thr Asp
                405                 410                 415
Leu His Ala Phe Glu Asn Leu Glu Ile Ile Arg Gly Arg Thr Lys Gln
            420                 425                 430
His Gly Gln Phe Ser Leu Ala Val Val Ser Leu Asn Ile Thr Ser Leu
        435                 440                 445
Gly Leu Arg Ser Leu Lys Glu Ile Ser Asp Gly Asp Val Ile Ile Ser
    450                 455                 460
Gly Asn Lys Asn Leu Cys Tyr Ala Asn Thr Ile Asn Trp Lys Lys Leu
465                 470                 475                 480
Phe Gly Thr Ser Gly Gln Lys Thr Lys Ile Ile Ser Asn Arg Gly Glu
                485                 490                 495
Asn Ser Cys Lys Ala Thr Gly Gln Val Cys His Ala Leu Cys Ser Pro
            500                 505                 510
Glu Gly Cys Trp Gly Pro Glu Pro Arg Asp Cys Val Ser Cys Arg Asn
        515                 520                 525
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Val Ser Arg Gly Arg Glu Cys Val Asp Lys Cys Asn Leu Leu Glu Gly
    530                 535                 540
Glu Pro Arg Glu Phe Val Glu Asn Ser Glu Cys Ile Gln Cys His Pro
545                 550                 555                 560
Glu Cys Leu Pro Gln Ala Met Asn Ile Thr Cys Thr Gly Arg Gly Pro
                565                 570                 575
Asp Asn Cys Ile Gln Cys Ala His Tyr Ile Asp Gly Pro His Cys Val
            580                 585                 590
Lys Thr Cys Pro Ala Gly Val Met Gly Glu Asn Asn Thr Leu Val Trp
        595                 600                 605
Lys Tyr Ala Asp Ala Gly His Val Cys His Leu Cys His Pro Asn Cys
    610                 615                 620
Thr Tyr Gly Cys Thr Gly Pro Gly Leu Glu Gly Cys Pro Thr Asn Gly
625                 630                 635                 640
Pro Lys Ile Pro Ser Ile Ala Thr Gly Met Val Gly Ala Leu Leu Leu
                645                 650                 655
Leu Leu Val Val Ala Leu Gly Ile Gly Leu Phe Met Arg Arg Arg His
            660                 665                 670
Ile Val Arg Lys Arg Thr Leu Arg Arg Leu Leu Gln Glu Arg Glu Leu
        675                 680                 685
Val Glu Pro Leu Thr Pro Ser Gly Glu Ala Pro Asn Gln Ala Leu Leu
    690                 695                 700
Arg Ile Leu Lys Glu Thr Glu Phe Lys Lys Ile Lys Val Leu Gly Ser
705                 710                 715                 720
Gly Ala Phe Gly Thr Val Tyr Lys Gly Leu Trp Ile Pro Glu Gly Glu
                725                 730                 735
Lys Val Lys Ile Pro Val Ala Ile Lys Glu Leu Arg Glu Ala Thr Ser
            740                 745                 750
Pro Lys Ala Asn Lys Glu Ile Leu Asp Glu Ala Tyr Val Met Ala Ser
        755                 760                 765
Val Asp Asn Pro His Val Cys Arg Leu Leu Gly Ile Cys Leu Thr Ser
    770                 775                 780
Thr Val Gln Leu Ile Thr Gln Leu Met Pro Phe Gly Cys Leu Leu Asp
785                 790                 795                 800
Tyr Val Arg Glu His Lys Asp Asn Ile Gly Ser Gln Tyr Leu Leu Asn
                805                 810                 815
Trp Cys Val Gln Ile Ala Lys Gly Met Asn Tyr Leu Glu Asp Arg Arg
            820                 825                 830
Leu Val His Arg Asp Leu Ala Ala Arg Asn Val Leu Val Lys Thr Pro
        835                 840                 845
Gln His Val Lys Ile Thr Asp Phe Gly Leu Ala Lys Leu Leu Gly Ala
    850                 855                 860
Glu Glu Lys Glu Tyr His Ala Glu Gly Gly Lys Val Pro Ile Lys Trp
865                 870                 875                 880
Met Ala Leu Glu Ser Ile Leu His Arg Ile Tyr Thr His Gln Ser Asp
                885                 890                 895
Val Trp Ser Tyr Gly Val Thr Val Trp Glu Leu Met Thr Phe Gly Ser
            900                 905                 910
Lys Pro Tyr Asp Gly Ile Pro Ala Ser Glu Ile Ser Ser Ile Leu Glu
        915                 920                 925
Lys Gly Glu Arg Leu Pro Gln Pro Pro Ile Cys Thr Ile Asp Val Tyr
    930                 935                 940
Met Ile Met Val Lys Cys Trp Met Ile Asp Ala Asp Ser Arg Pro Lys
945                 950                 955                 960
Phe Arg Glu Leu Ile Ile Glu Phe Ser Lys Met Ala Arg Asp Pro Gln
                965                 970                 975
Arg Tyr Leu Val Ile Gln Gly Asp Glu Arg Met His Leu Pro Ser Pro
            980                 985                 990
Thr Asp Ser Asn Phe Tyr Arg Ala Leu Met Asp Glu Glu Asp Met Asp
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        995                 1000                1005
Asp Val Val Asp Ala Asp Glu Tyr Leu Ile Pro Gln Gln Gly Phe Phe
    1010                1015                1020
Ser Ser Pro Ser Thr Ser Arg Thr Pro Leu Leu Ser Ser Leu Ser Ala
1025                1030                1035                1040
Thr Ser Asn Asn Ser Thr Val Ala Cys Ile Asp Arg Asn Gly Leu Gln
                1045                1050                1055
Ser Cys Pro Ile Lys Glu Asp Ser Phe Leu Gln Arg Tyr Ser Ser Asp
            1060                1065                1070
Pro Thr Gly Ala Leu Thr Glu Asp Ser Ile Asp Asp Thr Phe Leu Pro
        1075                1080                1085
Val Pro Glu Tyr Ile Asn Gln Ser Val Pro Lys Arg Pro Ala Gly Ser
    1090                1095                1100
Val Gln Asn Pro Val Tyr His Asn Gln Pro Leu Asn Pro Ala Pro Ser
1105                1110                1115                1120
Arg Asp Pro His Tyr Gln Asp Pro His Ser Thr Ala Val Gly Asn Pro
                1125                1130                1135
Glu Tyr Leu Asn Thr Val Gln Pro Thr Cys Val Asn Ser Thr Phe Asp
            1140                1145                1150
Ser Pro Ala His Trp Ala Gln Lys Gly Ser His Gln Ile Ser Leu Asp
        1155                1160                1165
Asn Pro Asp Tyr Gln Gln Asp Phe Phe Pro Lys Glu Ala Lys Pro Asn
    1170                1175                1180
Gly Ile Phe Lys Gly Ser Thr Ala Glu Asn Ala Glu Tyr Leu Arg Val
1185                1190                1195                1200
Ala Pro Gln Ser Ser Glu Phe Ile Gly Ala
                1205                1210

&lt;210&gt; 8
&lt;211&gt; 379
&lt;212&gt; PRT
&lt;213&gt; Homo Sapiens

&lt;400&gt; 8
Met Ala Ala Ala Ala Ala Gln Gly Gly Gly Gly Gly Glu Pro Arg Arg
 1              5                   10                  15
Thr Glu Gly Val Gly Pro Gly Val Pro Gly Glu Val Glu Met Val Lys
            20                  25                  30
Gly Gln Pro Phe Asp Val Gly Pro Arg Tyr Thr Gln Leu Gln Tyr Ile
        35                  40                  45
Gly Glu Gly Ala Tyr Gly Met Val Ser Ser Ala Tyr Asp His Val Arg
    50                  55                  60
Lys Thr Arg Val Ala Ile Lys Lys Ile Ser Pro Phe Glu His Gln Thr
65                  70                  75                  80
Tyr Cys Gln Arg Thr Leu Arg Glu Ile Gln Ile Leu Leu Arg Phe Arg
                85                  90                  95
His Glu Asn Val Ile Gly Ile Arg Asp Ile Leu Arg Ala Ser Thr Leu
            100                 105                 110
Glu Ala Met Arg Asp Val Tyr Ile Val Gln Asp Leu Met Glu Thr Asp
        115                 120                 125
Leu Tyr Lys Leu Leu Lys Ser Gln Gln Leu Ser Asn Asp His Ile Cys
    130                 135                 140
Tyr Phe Leu Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala
145                 150                 155                 160
Asn Val Leu His Arg Asp Leu Lys Pro Ser Asn Leu Leu Ile Asn Thr
                165                 170                 175
Thr Cys Asp Leu Lys Ile Cys Asp Phe Gly Leu Ala Arg Ile Ala Asp
            180                 185                 190
Pro Glu His Asp His Thr Gly Phe Leu Thr Glu Tyr Val Ala Thr Arg
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        195                 200                 205
Trp Tyr Arg Ala Pro Glu Ile Met Leu Asn Ser Lys Gly Tyr Thr Lys
    210                 215                 220
Ser Ile Asp Ile Trp Ser Val Gly Cys Ile Leu Ala Glu Met Leu Ser
225                 230                 235                 240
Asn Arg Pro Ile Phe Pro Gly Lys His Tyr Leu Asp Gln Leu Asn His
                245                 250                 255
Ile Leu Gly Ile Leu Gly Ser Pro Ser Gln Glu Asp Leu Asn Cys Ile
            260                 265                 270
Ile Asn Met Lys Ala Arg Asn Tyr Leu Gln Ser Leu Pro Ser Lys Thr
        275                 280                 285
Lys Val Ala Trp Ala Lys Leu Phe Pro Lys Ser Asp Ser Lys Ala Leu
    290                 295                 300
Asp Leu Leu Asp Arg Met Leu Thr Phe Asn Pro Asn Lys Arg Ile Thr
305                 310                 315                 320
Val Glu Glu Ala Leu Ala His Pro Tyr Leu Glu Gln Tyr Tyr Asp Pro
                325                 330                 335
Thr Asp Glu Pro Val Ala Glu Glu Pro Phe Thr Phe Ala Met Glu Leu
            340                 345                 350
Asp Asp Leu Pro Lys Glu Arg Leu Lys Glu Leu Ile Phe Gln Glu Thr
        355                 360                 365
Ala Arg Phe Gln Pro Gly Val Leu Glu Ala Pro
    370                 375

&lt;210&gt; 9
&lt;211&gt; 3256
&lt;212&gt; PRT
&lt;213&gt; Homo Sapiens

&lt;400&gt; 9
Met Trp Pro Thr Arg Arg Leu Val Thr Ile Lys Arg Ser Gly Val Asp
 1               5                  10                  15
Gly Pro His Phe Pro Leu Ser Leu Ser Thr Cys Leu Phe Gly Arg Gly
            20                  25                  30
Ile Glu Cys Asp Ile Arg Ile Gln Leu Pro Val Val Ser Lys Gln His
        35                  40                  45
Cys Lys Ile Glu Ile His Glu Gln Glu Ala Ile Leu His Asn Phe Ser
    50                  55                  60
Ser Thr Asn Pro Thr Gln Val Asn Gly Ser Val Ile Asp Glu Pro Val
65                  70                  75                  80
Arg Leu Lys His Gly Asp Val Ile Thr Ile Ile Asp Arg Ser Phe Arg
                85                  90                  95
Tyr Glu Asn Glu Ser Leu Gln Asn Gly Arg Lys Ser Thr Glu Phe Pro
            100                 105                 110
Arg Lys Ile Arg Glu Gln Glu Pro Ala Arg Arg Val Ser Arg Ser Ser
        115                 120                 125
Phe Ser Ser Asp Pro Asp Glu Lys Ala Gln Asp Ser Lys Ala Tyr Ser
    130                 135                 140
Lys Ile Thr Glu Gly Lys Val Ser Gly Asn Pro Gln Val His Ile Lys
145                 150                 155                 160
Asn Val Lys Glu Asp Ser Thr Ala Asp Asp Ser Lys Asp Ser Val Ala
                165                 170                 175
Gln Gly Thr Thr Asn Val His Ser Ser Glu His Ala Gly Arg Asn Gly
            180                 185                 190
Arg Asn Ala Ala Asp Pro Ile Ser Gly Asp Phe Lys Glu Ile Ser Ser
        195                 200                 205
Val Lys Leu Val Ser Arg Tyr Gly Glu Leu Lys Ser Val Pro Thr Thr
    210                 215                 220
Gln Cys Leu Asp Asn Ser Lys Lys Asn Glu Ser Pro Phe Trp Lys Leu
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225                 230                 235                 240
Tyr Glu Ser Val Lys Lys Glu Leu Asp Val Lys Ser Gln Lys Glu Asn
                245                 250                 255
Val Leu Gln Tyr Cys Arg Lys Ser Gly Leu Gln Thr Asp Tyr Ala Thr
            260                 265                 270
Glu Lys Glu Ser Ala Asp Gly Leu Gln Gly Glu Thr Gln Leu Leu Val
        275                 280                 285
Ser Arg Lys Ser Arg Pro Lys Ser Gly Gly Ser Gly His Ala Val Ala
    290                 295                 300
Glu Pro Ala Ser Pro Glu Gln Glu Leu Asp Gln Asn Lys Gly Lys Gly
305                 310                 315                 320
Arg Asp Val Glu Ser Val Gln Thr Pro Ser Lys Ala Val Gly Ala Ser
                325                 330                 335
Phe Pro Leu Tyr Glu Pro Ala Lys Met Lys Thr Pro Val Gln Tyr Ser
            340                 345                 350
Gln Gln Gln Asn Ser Pro Gln Lys His Lys Asn Lys Asp Leu Tyr Thr
        355                 360                 365
Thr Gly Arg Arg Glu Ser Val Asn Leu Gly Lys Ser Glu Gly Phe Lys
    370                 375                 380
Ala Gly Asp Lys Thr Leu Thr Pro Arg Lys Leu Ser Thr Arg Asn Arg
385                 390                 395                 400
Thr Pro Ala Lys Val Glu Asp Ala Ala Asp Ser Ala Thr Lys Pro Glu
                405                 410                 415
Asn Leu Ser Ser Lys Thr Arg Gly Ser Ile Pro Thr Asp Val Glu Val
            420                 425                 430
Leu Pro Thr Glu Thr Glu Ile His Asn Glu Pro Phe Leu Thr Leu Trp
        435                 440                 445
Leu Thr Gln Val Glu Arg Lys Ile Gln Lys Asp Ser Leu Ser Lys Pro
    450                 455                 460
Glu Lys Leu Gly Thr Thr Ala Gly Gln Met Cys Ser Gly Leu Pro Gly
465                 470                 475                 480
Leu Ser Ser Val Asp Ile Asn Asn Phe Gly Asp Ser Ile Asn Glu Ser
                485                 490                 495
Glu Gly Ile Pro Leu Lys Arg Arg Arg Val Ser Phe Gly Gly His Leu
            500                 505                 510
Arg Pro Glu Leu Phe Asp Glu Asn Leu Pro Pro Asn Thr Pro Leu Lys
        515                 520                 525
Arg Gly Glu Ala Pro Thr Lys Arg Lys Ser Leu Val Met His Thr Pro
    530                 535                 540
Pro Val Leu Lys Lys Ile Ile Lys Glu Gln Pro Gln Pro Ser Gly Lys
545                 550                 555                 560
Gln Glu Ser Gly Ser Glu Ile His Val Glu Val Lys Ala Gln Ser Leu
                565                 570                 575
Val Ile Ser Pro Pro Ala Pro Ser Pro Arg Lys Thr Pro Val Ala Ser
            580                 585                 590
Asp Gln Arg Arg Arg Ser Cys Lys Thr Ala Pro Ala Ser Ser Ser Lys
        595                 600                 605
Ser Gln Thr Glu Val Pro Lys Arg Gly Gly Glu Arg Val Ala Thr Cys
    610                 615                 620
Leu Gln Lys Arg Val Ser Ile Ser Arg Ser Gln His Asp Ile Leu Gln
625                 630                 635                 640
Met Ile Cys Ser Lys Arg Arg Ser Gly Ala Ser Glu Ala Asn Leu Ile
                645                 650                 655
Val Ala Lys Ser Trp Ala Asp Val Val Lys Leu Gly Ala Lys Gln Thr
            660                 665                 670
Gln Thr Lys Val Ile Lys His Gly Pro Gln Arg Ser Met Asn Lys Arg
        675                 680                 685
Gln Arg Arg Pro Ala Thr Pro Lys Lys Pro Val Gly Glu Val His Ser
    690                 695                 700
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Gln Phe Ser Thr Gly His Ala Asn Ser Pro Cys Thr Ile Ile Ile Gly
705                 710                 715                 720
Lys Ala His Thr Glu Lys Val His Val Pro Ala Arg Pro Tyr Arg Val
                725                 730                 735
Leu Asn Asn Phe Ile Ser Asn Gln Lys Met Asp Phe Lys Glu Asp Leu
            740                 745                 750
Ser Gly Ile Ala Glu Met Phe Lys Thr Pro Val Lys Glu Gln Pro Gln
        755                 760                 765
Leu Thr Ser Thr Cys His Ile Ala Ile Ser Asn Ser Glu Asn Leu Leu
    770                 775                 780
Gly Lys Gln Phe Gln Gly Thr Asp Ser Gly Glu Glu Pro Leu Leu Pro
785                 790                 795                 800
Thr Ser Glu Ser Phe Gly Gly Asn Val Phe Phe Ser Ala Gln Asn Ala
                805                 810                 815
Ala Lys Gln Pro Ser Asp Lys Cys Ser Ala Ser Pro Pro Leu Arg Arg
            820                 825                 830
Gln Cys Ile Arg Glu Asn Gly Asn Val Ala Lys Thr Pro Arg Asn Thr
        835                 840                 845
Tyr Lys Met Thr Ser Leu Glu Thr Lys Thr Ser Asp Thr Glu Thr Glu
    850                 855                 860
Pro Ser Lys Thr Val Ser Thr Val Asn Arg Ser Gly Arg Ser Thr Glu
865                 870                 875                 880
Phe Arg Asn Ile Gln Lys Leu Pro Val Glu Ser Lys Ser Glu Glu Thr
                885                 890                 895
Asn Thr Glu Ile Val Glu Cys Ile Leu Lys Arg Gly Gln Lys Ala Thr
            900                 905                 910
Leu Leu Gln Gln Arg Arg Glu Gly Glu Met Lys Glu Ile Glu Arg Pro
        915                 920                 925
Phe Glu Thr Tyr Lys Glu Asn Ile Glu Leu Lys Glu Asn Asp Glu Lys
    930                 935                 940
Met Lys Ala Met Lys Arg Ser Arg Thr Trp Gly Gln Lys Cys Ala Pro
945                 950                 955                 960
Met Ser Asp Leu Thr Asp Leu Lys Ser Leu Pro Asp Thr Glu Leu Met
                965                 970                 975
Lys Asp Thr Ala Arg Gly Gln Asn Leu Leu Gln Thr Gln Asp His Ala
            980                 985                 990
Lys Ala Pro Lys Ser Glu Lys Gly Lys Ile Thr Lys Met Pro Cys Gln
        995                 1000                1005
Ser Leu Gln Pro Glu Pro Ile Asn Thr Pro Thr His Thr Lys Gln Gln
    1010                1015                1020
Leu Lys Ala Ser Leu Gly Lys Val Gly Val Lys Glu Glu Leu Leu Ala
1025                1030                1035                1040
Val Gly Lys Phe Thr Arg Thr Ser Gly Glu Thr Thr His Thr His Arg
                1045                1050                1055
Glu Pro Ala Gly Asp Gly Lys Ser Ile Arg Thr Phe Lys Glu Ser Pro
            1060                1065                1070
Lys Gln Ile Leu Asp Pro Ala Ala Arg Val Thr Gly Met Lys Lys Trp
        1075                1080                1085
Pro Arg Thr Pro Lys Glu Glu Ala Gln Ser Leu Glu Asp Leu Ala Gly
    1090                1095                1100
Phe Lys Glu Leu Phe Gln Thr Pro Gly Pro Ser Glu Glu Ser Met Thr
1105                1110                1115                1120
Asp Glu Lys Thr Thr Lys Ile Ala Cys Lys Ser Pro Pro Pro Glu Ser
                1125                1130                1135
Val Asp Thr Pro Thr Ser Thr Lys Gln Trp Pro Lys Arg Ser Leu Arg
            1140                1145                1150
Lys Ala Asp Val Glu Glu Glu Phe Leu Ala Leu Arg Lys Leu Thr Pro
        1155                1160                1165
Ser Ala Gly Lys Ala Met Leu Thr Pro Lys Pro Ala Gly Gly Asp Glu
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    1170                1175                1180
Lys Asp Ile Lys Ala Phe Met Gly Thr Pro Val Gln Lys Leu Asp Leu
1185                1190                1195                1200
Ala Gly Thr Leu Pro Gly Ser Lys Arg Gln Leu Gln Thr Pro Lys Glu
                1205                1210                1215
Lys Ala Gln Ala Leu Glu Asp Leu Ala Gly Phe Lys Glu Leu Phe Gln
            1220                1225                1230
Thr Pro Gly His Thr Glu Glu Leu Val Ala Ala Gly Lys Thr Thr Lys
        1235                1240                1245
Ile Pro Cys Asp Ser Pro Gln Ser Asp Pro Val Asp Thr Pro Thr Ser
    1250                1255                1260
Thr Lys Gln Arg Pro Lys Arg Ser Ile Arg Lys Ala Asp Val Glu Gly
1265                1270                1275                1280
Glu Leu Leu Ala Cys Arg Asn Leu Met Pro Ser Ala Gly Lys Ala Met
                1285                1290                1295
His Thr Pro Lys Pro Ser Val Gly Glu Glu Lys Asp Ile Ile Ile Phe
            1300                1305                1310
Val Gly Thr Pro Val Gln Lys Leu Asp Leu Thr Glu Asn Leu Thr Gly
        1315                1320                1325
Ser Lys Arg Arg Pro Gln Thr Pro Lys Glu Glu Ala Gln Ala Leu Glu
    1330                1335                1340
Asp Leu Thr Gly Phe Lys Glu Leu Phe Gln Thr Pro Gly His Thr Glu
1345                1350                1355                1360
Glu Ala Val Ala Ala Gly Lys Thr Thr Lys Met Pro Cys Glu Ser Ser
                1365                1370                1375
Pro Pro Glu Ser Ala Asp Thr Pro Thr Ser Thr Arg Arg Gln Pro Lys
            1380                1385                1390
Thr Pro Leu Glu Lys Arg Asp Val Gln Lys Glu Leu Ser Ala Leu Lys
        1395                1400                1405
Lys Leu Thr Gln Thr Ser Gly Glu Thr Thr His Thr Asp Lys Val Pro
    1410                1415                1420
Gly Gly Glu Asp Lys Ser Ile Asn Ala Phe Arg Glu Thr Ala Lys Gln
1425                1430                1435                1440
Lys Leu Asp Pro Ala Ala Ser Val Thr Gly Ser Lys Arg His Pro Lys
                1445                1450                1455
Thr Lys Glu Lys Ala Gln Pro Leu Glu Asp Leu Ala Gly Trp Lys Glu
            1460                1465                1470
Leu Phe Gln Thr Pro Val Cys Thr Asp Lys Pro Thr Thr His Glu Lys
        1475                1480                1485
Thr Thr Lys Ile Ala Cys Arg Ser Gln Pro Asp Pro Val Asp Thr Pro
    1490                1495                1500
Thr Ser Ser Lys Pro Gln Ser Lys Arg Ser Leu Arg Lys Val Asp Val
1505                1510                1515                1520
Glu Glu Glu Phe Phe Ala Leu Arg Lys Arg Thr Pro Ser Ala Gly Lys
                1525                1530                1535
Ala Met His Thr Pro Lys Pro Ala Val Ser Gly Glu Lys Asn Ile Tyr
            1540                1545                1550
Ala Phe Met Gly Thr Pro Val Gln Lys Leu Asp Leu Thr Glu Asn Leu
        1555                1560                1565
Thr Gly Ser Lys Arg Arg Leu Gln Thr Pro Lys Glu Lys Ala Gln Ala
    1570                1575                1580
Leu Glu Asp Leu Ala Gly Phe Lys Glu Leu Phe Gln Thr Arg Gly His
1585                1590                1595                1600
Thr Glu Glu Ser Met Thr Asn Asp Lys Thr Ala Lys Val Ala Cys Lys
                1605                1610                1615
Ser Ser Gln Pro Asp Leu Asp Lys Asn Pro Ala Ser Ser Lys Arg Arg
            1620                1625                1630
Leu Lys Thr Ser Leu Gly Lys Val Gly Val Lys Glu Glu Leu Leu Ala
        1635                1640                1645
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Val Gly Lys Leu Thr Gln Thr Ser Gly Glu Thr Thr His Thr His Thr
    1650                1655                1660
Glu Pro Thr Gly Asp Gly Lys Ser Met Lys Ala Phe Met Glu Ser Pro
1665                1670                1675                1680
Lys Gln Ile Leu Asp Ser Ala Ala Ser Leu Thr Gly Ser Lys Arg Gln
                1685                1690                1695
Leu Arg Thr Pro Lys Gly Lys Ser Glu Val Pro Glu Asp Leu Ala Gly
            1700                1705                1710
Phe Ile Glu Leu Phe Gln Thr Pro Ser His Thr Lys Glu Ser Met Thr
        1715                1720                1725
Asn Glu Lys Thr Thr Lys Val Ser Tyr Arg Ala Ser Gln Pro Asp Leu
    1730                1735                1740
Val Asp Thr Pro Thr Ser Ser Lys Pro Gln Pro Lys Arg Ser Leu Arg
1745                1750                1755                1760
Lys Ala Asp Thr Glu Glu Glu Phe Leu Ala Phe Arg Lys Gln Thr Pro
                1765                1770                1775
Ser Ala Gly Lys Ala Met His Thr Pro Lys Pro Ala Val Gly Glu Glu
            1780                1785                1790
Lys Asp Ile Asn Thr Phe Leu Gly Thr Pro Val Gln Lys Leu Asp Gln
        1795                1800                1805
Pro Gly Asn Leu Pro Gly Ser Asn Arg Arg Leu Gln Thr Arg Lys Glu
    1810                1815                1820
Lys Ala Gln Ala Leu Glu Glu Leu Thr Gly Phe Arg Glu Leu Phe Gln
1825                1830                1835                1840
Thr Pro Cys Thr Asp Asn Pro Thr Ala Asp Glu Lys Thr Thr Lys Lys
                1845                1850                1855
Ile Leu Cys Lys Ser Pro Gln Ser Asp Pro Ala Asp Thr Pro Thr Asn
            1860                1865                1870
Thr Lys Gln Arg Pro Lys Arg Ser Leu Lys Lys Ala Asp Val Glu Glu
        1875                1880                1885
Glu Phe Leu Ala Phe Arg Lys Leu Thr Pro Ser Ala Gly Lys Ala Met
    1890                1895                1900
His Thr Pro Lys Ala Ala Val Gly Glu Glu Lys Asp Ile Asn Thr Phe
1905                1910                1915                1920
Val Gly Thr Pro Val Glu Lys Leu Asp Leu Leu Gly Asn Leu Pro Gly
                1925                1930                1935
Ser Lys Arg Arg Pro Gln Thr Pro Lys Glu Lys Ala Lys Ala Leu Glu
            1940                1945                1950
Asp Leu Ala Gly Phe Lys Glu Leu Phe Gln Thr Pro Gly His Thr Glu
        1955                1960                1965
Glu Ser Met Thr Asp Asp Lys Ile Thr Glu Val Ser Cys Lys Ser Pro
    1970                1975                1980
Gln Pro Asp Pro Val Lys Thr Pro Thr Ser Ser Lys Gln Arg Leu Lys
1985                1990                1995                2000
Ile Ser Leu Gly Lys Val Gly Val Lys Glu Glu Val Leu Pro Val Gly
                2005                2010                2015
Lys Leu Thr Gln Thr Ser Gly Lys Thr Thr Gln Thr His Arg Glu Thr
            2020                2025                2030
Ala Gly Asp Gly Lys Ser Ile Lys Ala Phe Lys Glu Ser Ala Lys Gln
        2035                2040                2045
Met Leu Asp Pro Ala Asn Tyr Gly Thr Gly Met Glu Arg Trp Pro Arg
    2050                2055                2060
Thr Pro Lys Glu Glu Ala Gln Ser Leu Glu Asp Leu Ala Gly Phe Lys
2065                2070                2075                2080
Glu Leu Phe Gln Thr Pro Asp His Thr Glu Glu Ser Thr Thr Asp Asp
                2085                2090                2095
Lys Thr Thr Lys Ile Ala Cys Lys Ser Pro Pro Pro Glu Ser Met Asp
            2100                2105                2110
Thr Pro Thr Ser Thr Arg Arg Arg Pro Lys Thr Pro Leu Gly Lys Arg
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        2115                2120                2125
Asp Ile Val Glu Glu Leu Ser Ala Leu Lys Gln Leu Thr Gln Thr Thr
    2130                2135                2140
His Thr Asp Lys Val Pro Gly Asp Glu Asp Lys Gly Ile Asn Val Phe
2145                2150                2155                2160
Arg Glu Thr Ala Lys Gln Lys Leu Asp Pro Ala Ala Ser Val Thr Gly
                2165                2170                2175
Ser Lys Arg Gln Pro Arg Thr Pro Lys Gly Lys Ala Gln Pro Leu Glu
            2180                2185                2190
Asp Leu Ala Gly Leu Lys Glu Leu Phe Gln Thr Pro Val Cys Thr Asp
        2195                2200                2205
Lys Pro Thr Thr His Glu Lys Thr Thr Lys Ile Ala Cys Arg Ser Pro
    2210                2215                2220
Gln Pro Asp Pro Val Gly Thr Pro Thr Ile Phe Lys Pro Gln Ser Lys
2225                2230                2235                2240
Arg Ser Leu Arg Lys Ala Asp Val Glu Glu Glu Ser Leu Ala Leu Arg
                2245                2250                2255
Lys Arg Thr Pro Ser Val Gly Lys Ala Met Asp Thr Pro Lys Pro Ala
            2260                2265                2270
Gly Gly Asp Glu Lys Asp Met Lys Ala Phe Met Gly Thr Pro Val Gln
        2275                2280                2285
Lys Leu Asp Leu Pro Gly Asn Leu Pro Gly Ser Lys Arg Trp Pro Gln
    2290                2295                2300
Thr Pro Lys Glu Lys Ala Gln Ala Leu Glu Asp Leu Ala Gly Phe Lys
2305                2310                2315                2320
Glu Leu Phe Gln Thr Pro Gly Thr Asp Lys Pro Thr Thr Asp Glu Lys
                2325                2330                2335
Thr Thr Lys Ile Ala Cys Lys Ser Pro Gln Pro Asp Pro Val Asp Thr
            2340                2345                2350
Pro Ala Ser Thr Lys Gln Arg Pro Lys Arg Asn Leu Arg Lys Ala Asp
        2355                2360                2365
Val Glu Glu Glu Phe Leu Ala Leu Arg Lys Arg Thr Pro Ser Ala Gly
    2370                2375                2380
Lys Ala Met Asp Thr Pro Lys Pro Ala Val Ser Asp Glu Lys Asn Ile
2385                2390                2395                2400
Asn Thr Phe Val Glu Thr Pro Val Gln Lys Leu Asp Leu Leu Gly Asn
                2405                2410                2415
Leu Pro Gly Ser Lys Arg Gln Pro Gln Thr Pro Lys Glu Lys Ala Glu
            2420                2425                2430
Ala Leu Glu Asp Leu Val Gly Phe Lys Glu Leu Phe Gln Thr Pro Gly
        2435                2440                2445
His Thr Glu Glu Ser Met Thr Asp Asp Lys Ile Thr Glu Val Ser Cys
    2450                2455                2460
Lys Ser Pro Gln Pro Glu Ser Phe Lys Thr Ser Arg Ser Ser Lys Gln
2465                2470                2475                2480
Arg Leu Lys Ile Pro Leu Val Lys Val Asp Met Lys Glu Glu Pro Leu
                2485                2490                2495
Ala Val Ser Lys Leu Thr Arg Thr Ser Gly Glu Thr Thr Gln Thr His
            2500                2505                2510
Thr Glu Pro Thr Gly Asp Ser Lys Ser Ile Lys Ala Phe Lys Glu Ser
        2515                2520                2525
Pro Lys Gln Ile Leu Asp Pro Ala Ala Ser Val Thr Gly Ser Arg Arg
    2530                2535                2540
Gln Leu Arg Thr Arg Lys Glu Lys Ala Arg Ala Leu Glu Asp Leu Val
2545                2550                2555                2560
Asp Phe Lys Glu Leu Phe Ser Ala Pro Gly His Thr Glu Glu Ser Met
                2565                2570                2575
Thr Ile Asp Lys Asn Thr Lys Ile Pro Cys Lys Ser Pro Pro Pro Glu
            2580                2585                2590
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Leu Thr Asp Thr Ala Thr Ser Thr Lys Arg Cys Pro Lys Thr Arg Pro
        2595                2600                2605
Arg Lys Glu Val Lys Glu Glu Leu Ser Ala Val Glu Arg Leu Thr Gln
    2610                2615                2620
Thr Ser Gly Gln Ser Thr His Thr His Lys Glu Pro Ala Ser Gly Asp
2625                2630                2635                2640
Glu Gly Ile Lys Val Leu Lys Gln Arg Ala Lys Lys Lys Pro Asn Pro
                2645                2650                2655
Val Glu Glu Glu Pro Ser Arg Arg Arg Pro Arg Ala Pro Lys Glu Lys
            2660                2665                2670
Ala Gln Pro Leu Glu Asp Leu Ala Gly Phe Thr Glu Leu Ser Glu Thr
        2675                2680                2685
Ser Gly His Thr Gln Glu Ser Leu Thr Ala Gly Lys Ala Thr Lys Ile
    2690                2695                2700
Pro Cys Glu Ser Pro Pro Leu Glu Val Val Asp Thr Thr Ala Ser Thr
2705                2710                2715                2720
Lys Arg His Leu Arg Thr Arg Val Gln Lys Val Gln Val Lys Glu Glu
                2725                2730                2735
Pro Ser Ala Val Lys Phe Thr Gln Thr Ser Gly Glu Thr Thr Asp Ala
            2740                2745                2750
Asp Lys Glu Pro Ala Gly Glu Asp Lys Gly Ile Lys Ala Leu Lys Glu
        2755                2760                2765
Ser Ala Lys Gln Thr Pro Ala Pro Ala Ala Ser Val Thr Gly Ser Arg
    2770                2775                2780
Arg Arg Pro Arg Ala Pro Arg Glu Ser Ala Gln Ala Ile Glu Asp Leu
2785                2790                2795                2800
Ala Gly Phe Lys Asp Pro Ala Ala Gly His Thr Glu Glu Ser Met Thr
                2805                2810                2815
Asp Asp Lys Thr Thr Lys Ile Pro Cys Lys Ser Ser Pro Glu Leu Glu
            2820                2825                2830
Asp Thr Ala Thr Ser Ser Lys Arg Arg Pro Arg Thr Arg Ala Gln Lys
        2835                2840                2845
Val Glu Val Lys Glu Glu Leu Leu Ala Val Gly Lys Leu Thr Gln Thr
    2850                2855                2860
Ser Gly Glu Thr Thr His Thr Asp Lys Glu Pro Val Gly Glu Gly Lys
2865                2870                2875                2880
Gly Thr Lys Ala Phe Lys Gln Pro Ala Lys Arg Asn Val Asp Ala Glu
                2885                2890                2895
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Ser Gly Thr Asp Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn
65                  70                  75                  80
Leu Lys Tyr Glu Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile
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Val Glu Leu Met Arg Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser
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Ser Phe Val Cys Val Leu Leu Ser His Gly Glu Glu Gly Ile Ile Phe
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His Ala Lys Lys Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/037288

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, WPI Data, PAJ, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/044218 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA; CELL SIGNALING TECHNOLOGY) 27 May 2004 (2004-05-27) the whole document -----	1-26
A	WO 03/087761 A (CELL SIGNALING TECHNOLOGY, INC; CROSBY, KATHERINE; SMITH, BRADLEY) 23 October 2003 (2003-10-23) example 2 -----	1-26

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 2005	12/08/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	van der Kooij, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PANIGRAHI A R ET AL: "The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome" JOURNAL OF PATHOLOGY, vol. 204, no. 1, September 2004 (2004-09), pages 93-100, XP008050606 ISSN: 0022-3417 abstract	1-26
A	XU GUANG ET AL: "Pharmacogenomic profiling of the PI3K/PTEN-AKT-mTOR pathway in common human tumors." INTERNATIONAL JOURNAL OF ONCOLOGY. APR 2004, vol. 24, no. 4, April 2004 (2004-04), pages 893-900, XP008050608 ISSN: 1019-6439 page 897, column 1, paragraph 3 - column 2, paragraph 2 page 898, column 2, paragraph 4	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US2004/037288

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004044218 A	27-05-2004	AU 2003291736 A1	03-06-2004
		WO 2004044218 A2	27-05-2004
		US 2004106141 A1	03-06-2004
WO 03087761 A	23-10-2003	AU 2003223495 A1	27-10-2003
		WO 03087761 A2	23-10-2003
		US 2003190689 A1	09-10-2003
		US 2004248151 A1	09-12-2004