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(54) **DIAGNOSIS AND TREATMENT OF  
MALIGNANT NEOPLASMS**

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continuation-in-part of application No. 09/436,184,  
filed on Nov. 8, 1999.

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(57) **ABSTRACT**

The invention features a method for diagnosing and inhibiting growth of a malignant neoplasm in a mammal by contacting a cell or a bodily fluid of the mammal with an antibody which binds to an human aspartyl (asparaginy) beta-hydroxylase (HAAH) polypeptide. Methods of immunization to generate an HAAH-specific immune response are also within the invention.

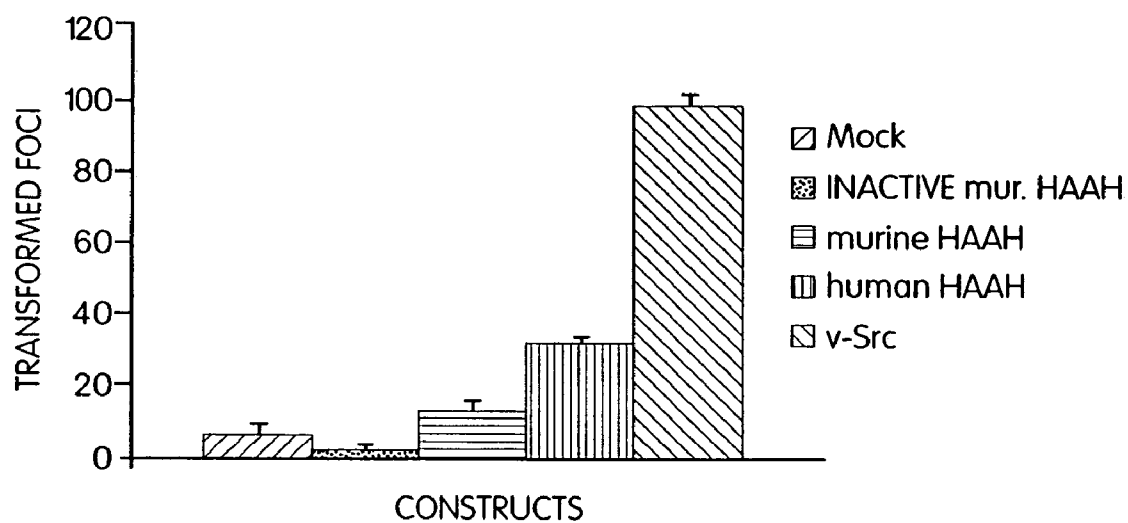


Fig. 1

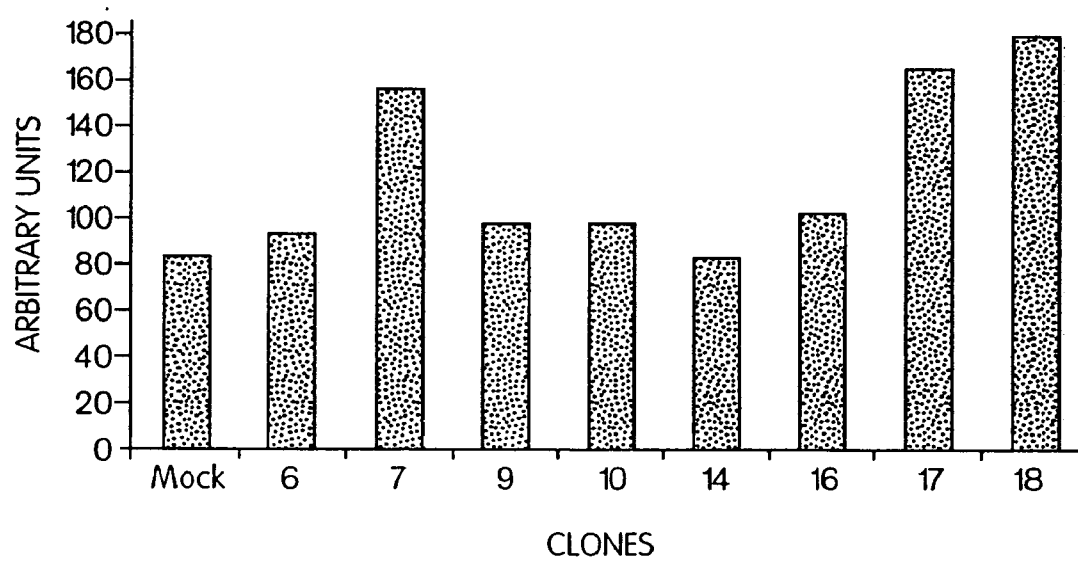


Fig. 2

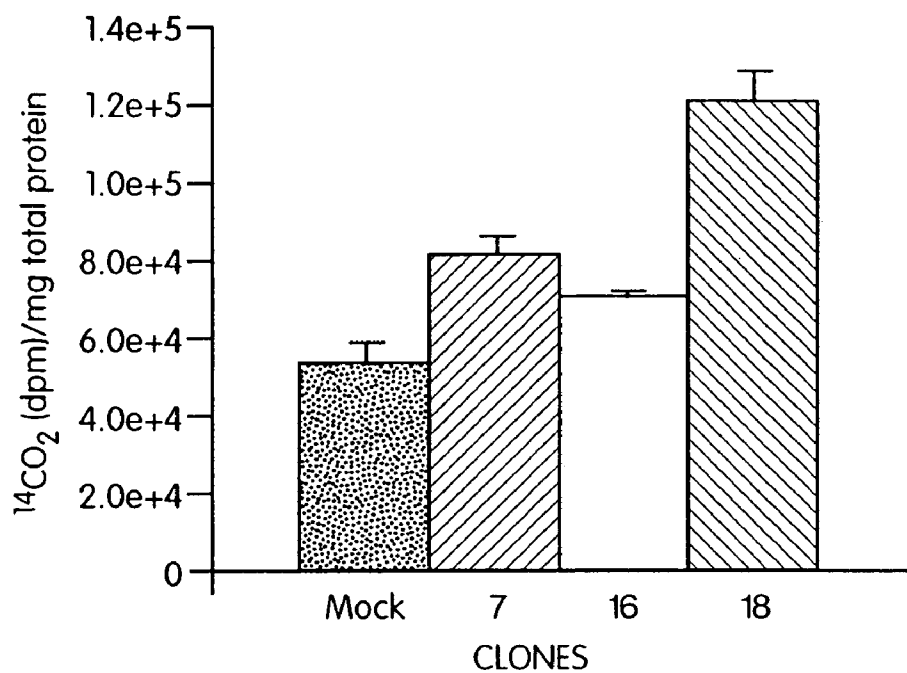


Fig. 3A

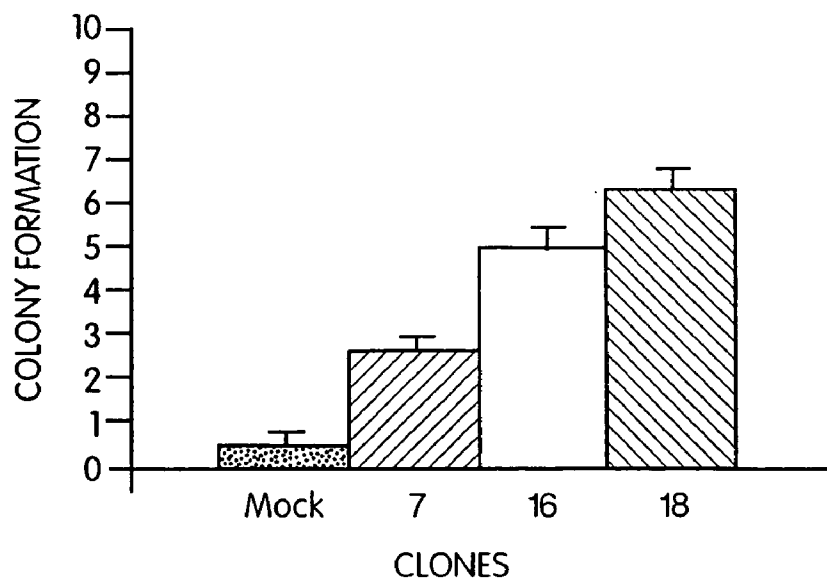


Fig. 3B

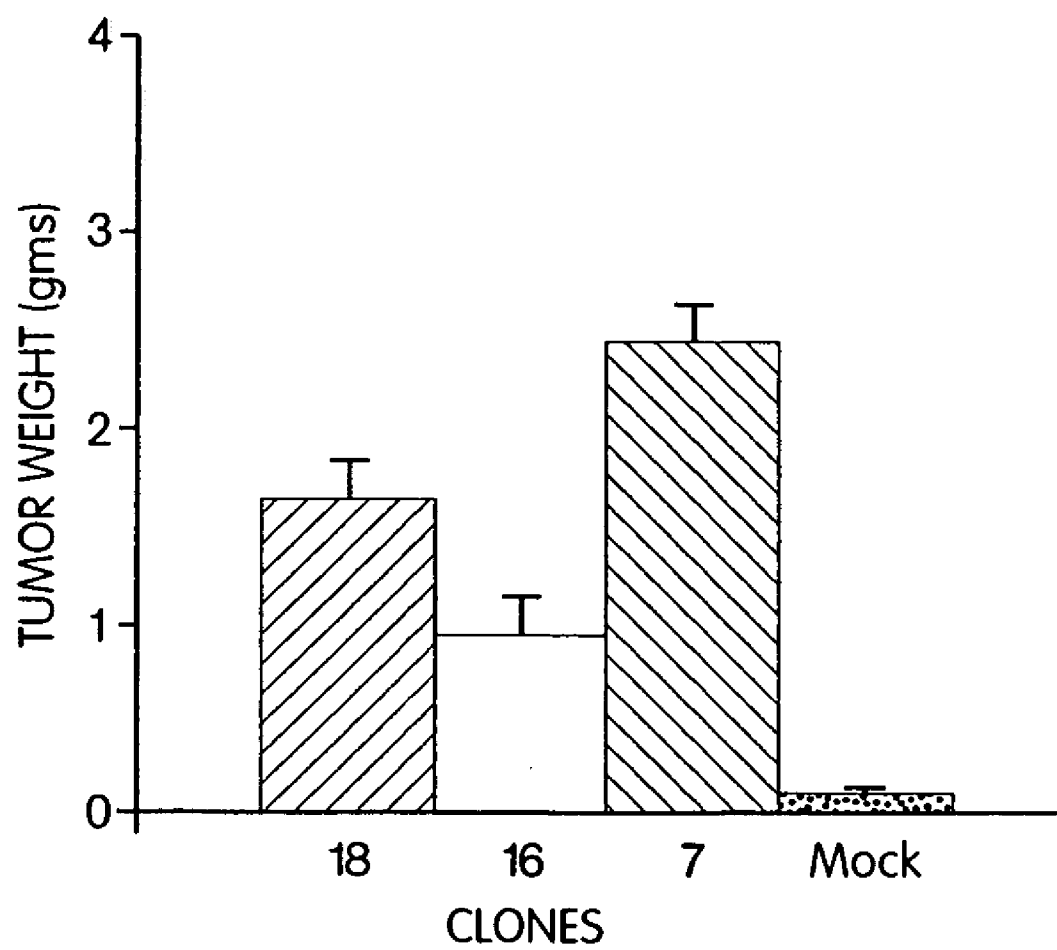


Fig. 4

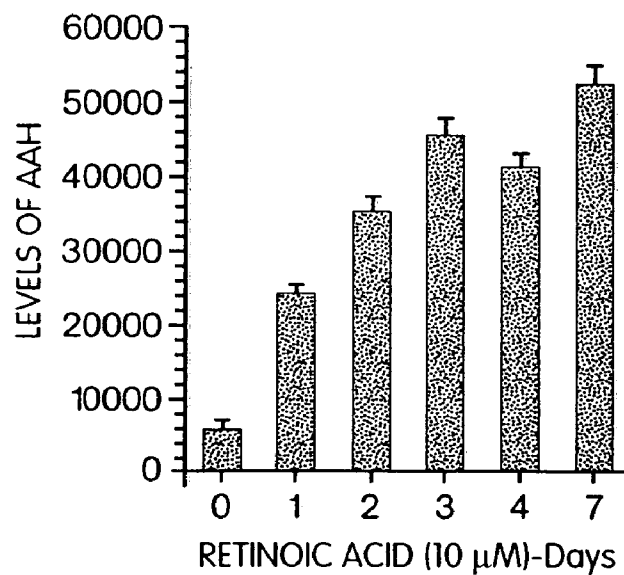


Fig. 5A

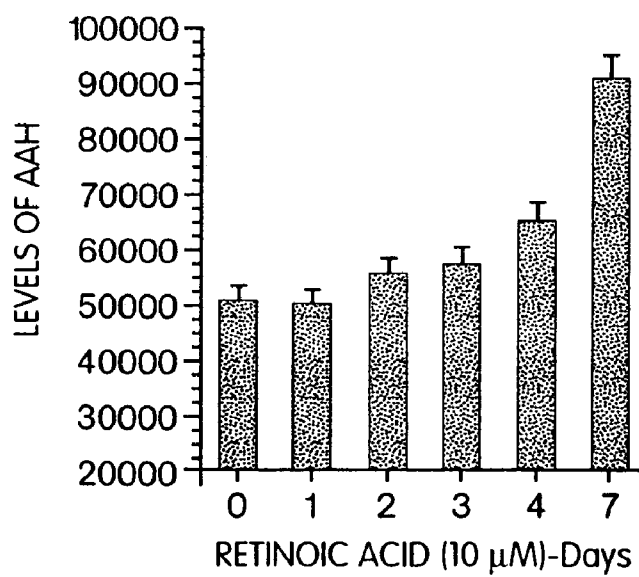


Fig. 5B

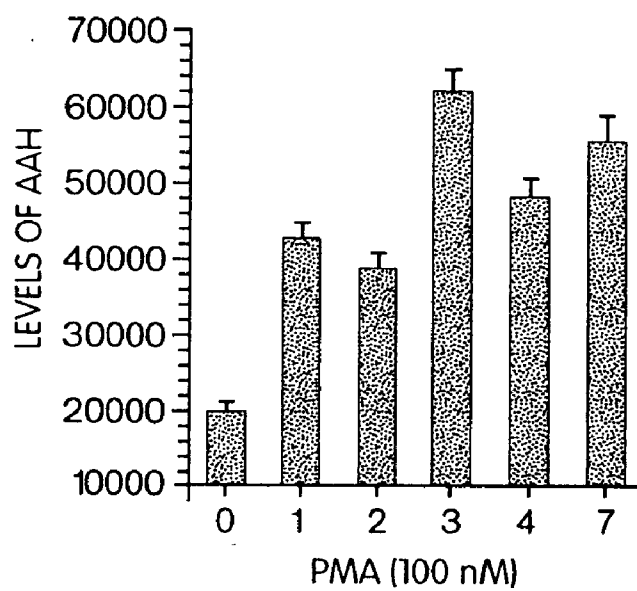


Fig. 5C

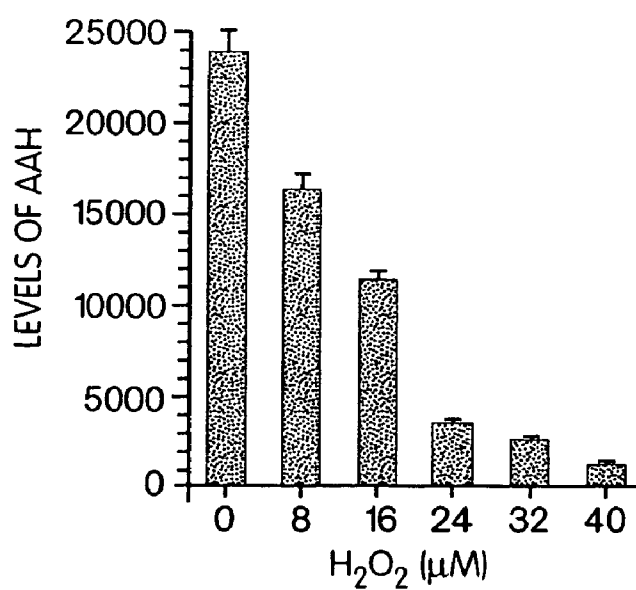


Fig. 5D

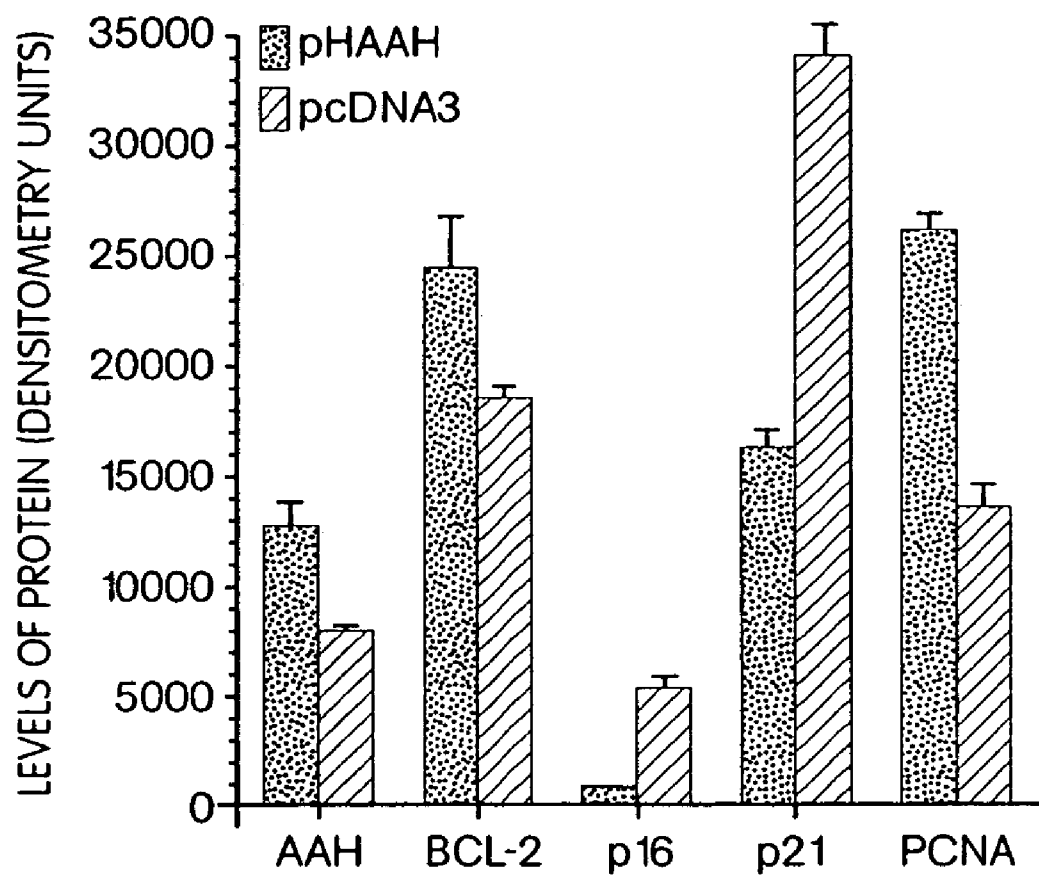


Fig. 6

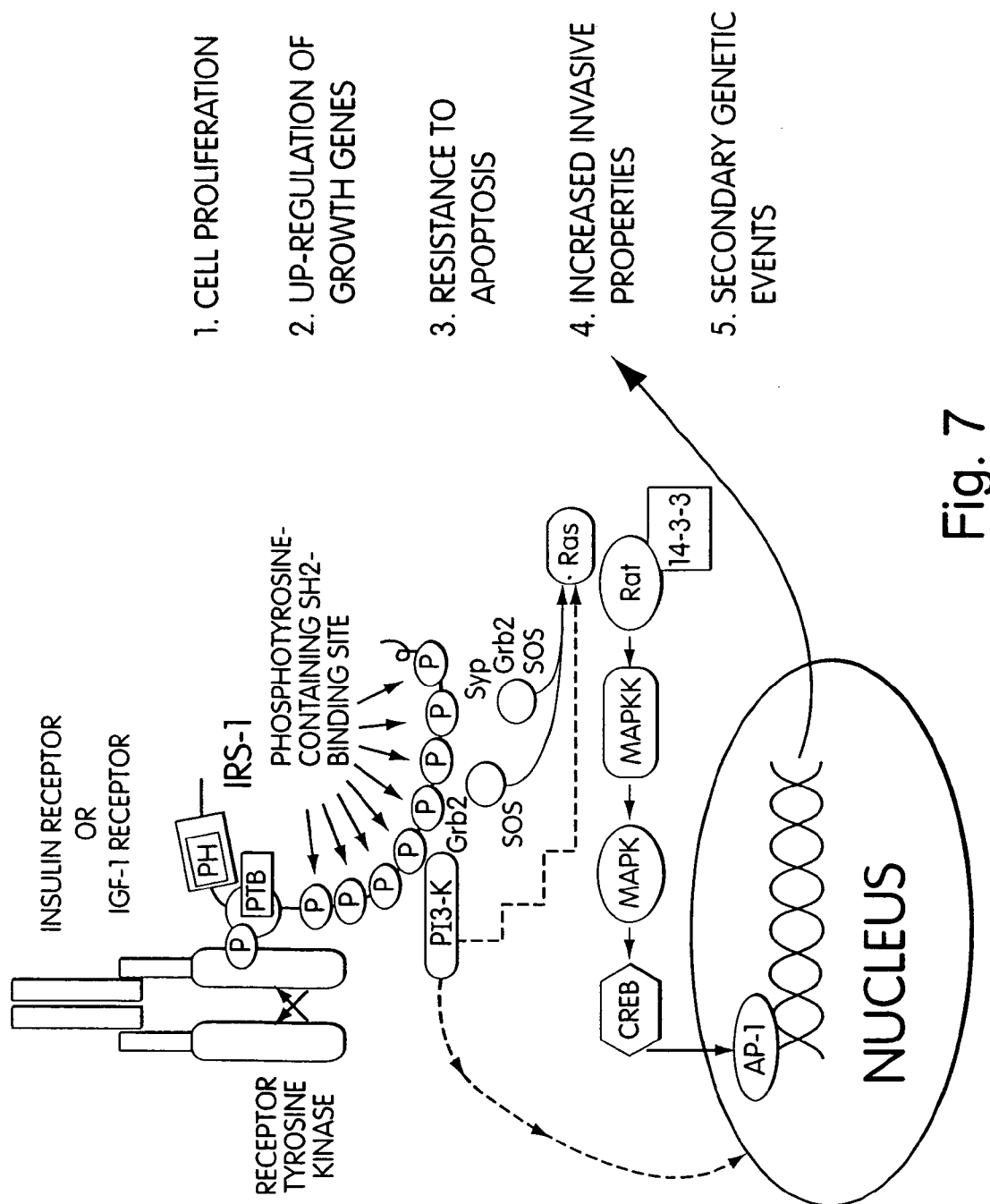


Fig. 7

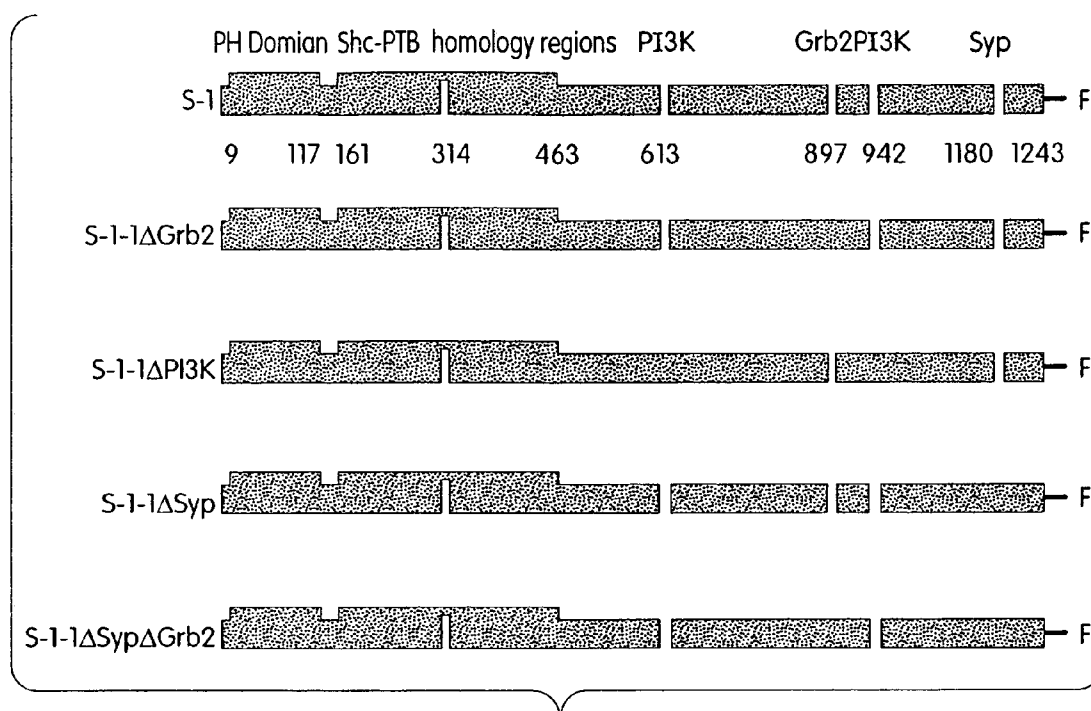


Fig. 8

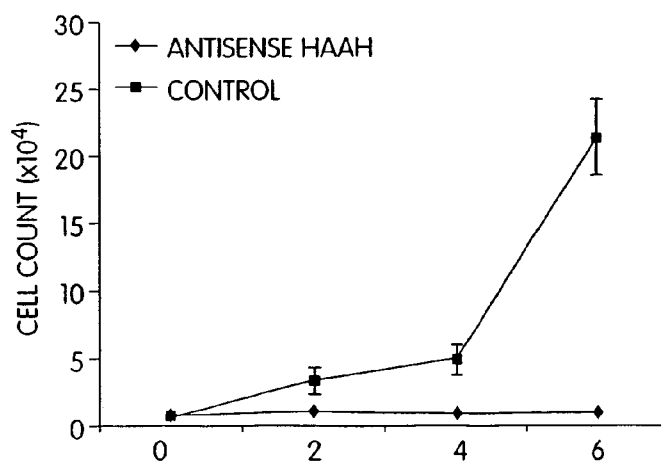


Fig. 9

## DIAGNOSIS AND TREATMENT OF MALIGNANT NEOPLASMS

### STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0001] This invention was made with U.S. Government support under National Institutes of Health grants CA-35711, AA-02666, AA-02169, and AA11431. The government has certain rights in the invention.

[0002] This application claims priority to patent application U.S. Ser. No. 09/436,184, filed on Nov. 8, 1999, and to U.S. Ser. No. 09/859,604, filed on May 17, 2001 the contents of which are hereby incorporated by reference.

### BACKGROUND OF THE INVENTION

[0003] Primary malignant central nervous system (CNS) neoplasms, particularly glioblastomas, are highly fatal due to their aggressive and widespread infiltration of the brain and resistance to anti-cancer treatments. Although progress has been made in unraveling the pathological mechanisms underlying CNS cancers as well as other cancer types, tumor specific therapeutic approaches and methods of diagnosis have been largely elusive.

### SUMMARY OF THE INVENTION

[0004] The invention features a method for diagnosing or inhibiting the growth of a malignant neoplasm in a mammal by contacting a tissue or bodily fluid from the mammal with an antibody which binds to an human aspartyl (asparaginy) beta-hydroxylase (HAAH) polypeptide under conditions sufficient to form an antigen-antibody complex and detecting the antigen-antibody complex. Malignant neoplasms detected in this manner include those derived from endodermal tissue, e.g., colon cancer, breast cancer, pancreatic cancer, liver cancer, and cancer of the bile ducts. Neoplasms of the central nervous system (CNS) such as primary malignant CNS neoplasms of both neuronal and glial cell origin and metastatic CNS neoplasms are also detected. Brain cancers include metastatic brain tumors, as well as primary brain tumors such as glioma, astrocytomas, and hemangiomas. Patient derived tissue samples, e.g., biopsies of solid tumors, as well as bodily fluids such as a CNS-derived bodily fluid, blood, serum, urine, saliva, sputum, lung effusion, and ascites fluid, are contacted with an HAAH-specific antibody.

[0005] The invention includes a method of eliciting an immune response or conferring an immune response to a tumor cell, e.g., a brain tumor, in a mammal by administering to a mammal an antibody, which binds to HAAH or a polynucleotide encoding such an antibody. Preferably, the antibody binds to a site in an extracellular domain (e.g., a site within residues 1-700) of HAAH. The antibody binds to an ectodomain of HAAH (residues 19-75 of SEQ ID NO:2). More preferably, the antibody binds to a catalytic domain of HAAH, e.g., amino acids 650-700 of SEQ ID NO:2. For example, FB50 binds to a polypeptide with the amino acid sequence NPVEDS (residues 286-291 of SEQ ID NO:2). Monoclonal antibody HBOH1 binds to a polypeptide with the amino acid sequence QPWWTPK (residues 573-579 of SEQ ID NO:2), and monoclonal antibody HBOH-2 binds to a polypeptide containing the amino acid sequence LPEDENLR (residues 613-620 of SEQ ID NO:2). The

foregoing antigenic epitopes of HAAH are located on the cell surface of malignant cells. Other HAAH-specific antibodies suitable for passive immunization include 5C7, 5E9, 19B, 48A, 74A, 78A, 86A, HA238A, HA221, HA239, HA241, HA329, and HA355.

[0006] The antibody to be administered is a heterodimeric antibody, a single chain antibody, or a high affinity single chain antibody. By high affinity is meant that the antigen-specific binding affinity of the antibody has a  $K_d$  in the nanomolar range. Preferably, the binding affinity is in the range of 100 pM or higher affinity. For example, the antibody, antibody fragment, or single chain antibody has an antigen-specific binding affinity in the range of  $10^{-10}$  to  $10^{-11}$  molar.

[0007] The antibody, or fragment thereof, activates complement in a patient treated with the antibody. Preferably, the antibody mediates antibody-dependent cytotoxicity of tumor cells in the patient treated with the antibody. The antibody, or fragment thereof, is administered alone or conjugated to a cytotoxic agent. In the latter case, binding of the antibody to a tumor cell results in impairment or death of the cell, thereby reducing tumor load. The antibody is conjugated to a radiochemical, or a chemical tag which sensitizes the cell to which it is bound to radiation or laser-mediated killing.

[0008] Also within the invention, are methods of inducing an HAAH-specific immune response to reduce tumor growth by active immunization. The method involves administering to a mammal an HAAH polypeptide, e.g., a polypeptide containing the amino acid sequence of SEQ ID NO:2. Immunogenic HAAH fragments are also administered to generate an immune response to a particular portion of HAAH. For example, to generate an antibody response to HAAH on the surface of cells, a polypeptide containing an extracellular domain of HAAH (but lacking an intracellular domain of HAAH) is administered. To generate antibodies, which inhibit HAAH activity, the individual is immunized with a polypeptide containing a catalytic domain of HAAH (e.g., amino acids 650-700 of SEQ ID NO:2). Optionally, the polypeptide compositions contain a clinically-acceptable adjuvant compound. Such adjuvants are generally known in the art, and include oil-emulsions, Freund's Complete and Incomplete adjuvant, Vitamin E, aluminum salts or gels, such as aluminum hydroxide, -oxide or -phosphate, saponins, polymers based on polyacrylic acid, such as carbopols, non-ionic block polymers, fatty acid amines, such as avidin and DDA, polymers based on dextran, such as dextran sulphate and DEAE dextran, muramyl dipeptides, ISCOMs (immune stimulating complexes, e.g., as described in European Patent EP 109942), biodegradable microcapsules, liposomes, bacterial immune stimulators, such as MDP and LPS, and glucans. Other adjuvant compounds are known in the art, e.g., described in Altman and Dixon, 1989, *Advances in Veterinary Science and Comparative Medicine* 33: 301-343). Alum is preferred for human use.

[0009] An HAAH-specific immune response is also induced by administering to a mammal a polynucleotide composition encoding an HAAH polypeptide, or a degenerate variant of the HAAH-encoding polynucleotide. For example, the polynucleotide contains the nucleotide sequence of SEQ ID NO:3, or a degenerate variant thereof, or a fragment thereof encoding a specific immunogenic

domain of HAAH. Preferably, the HAAH polypeptide encoded by the polynucleotide (or directly administered polypeptide) is enzymatically nonfunctional. More preferably, the HAAH polynucleotide encodes an HAAH polypeptide that is secreted, e.g., the construct contains a signal sequence for transport out of the cell and into an extracellular space. The HAAH polypeptide lacks an essential histidine. The HAAH polypeptide is a truncated HAAH, which contains the first 650 amino acids of SEQ ID NO:2.

[0010] Optionally, the polynucleotide composition contains a transfection-enhancing agent, such as a precipitating agent or a lipid. Preferably, the encoded HAAH polypeptide contains the amino acid sequence of SEQ ID NO:2 (full-length HAAH) or a fragment thereof, which contains an extracellular domain of HAAH and lacks an intracellular domain of HAAH. Preferably, the polynucleotide contains a catalytic domain of HAAH. The HAAH-encoding sequences are operably-linked to a promoter and other regulatory sequences for expression of the polypeptides in target cells. The polypeptide may be directed intracellularly or marked for extracellular expression, or secretion. The polynucleotide directs expression in a target cell, which expresses appropriate accessory molecules for antigen presentation, e.g., major histocompatibility antigens.

[0011] Methods for diagnosis include detecting a tumor cell in bodily fluids as well as detecting a tumor cell in tissue (in vivo or ex vivo). For example, a biopsied tissue is contacted with an HAAH-specific antibody and antibody binding measured. Whole body diagnostic imaging may be carried out to detect microtumors undetectable using conventional diagnostic methods. Accordingly, a method for diagnosing a neoplasm in a mammal is carried out by contacting a tissue, e.g., a lymph node, of a mammal with a detectably-labeled antibody which binds to HAAH. An increase in the level of antibody binding at a tissue site compared to the level of binding to a normal nonneoplastic tissue indicates the presence of a neoplasm at the tissue site. For detection purposes, the antibody (or HAAH-binding fragment thereof) is labeled with a non-radioactive tag, a radioactive compound, or a colorimetric agent. For example, the antibody or antibody fragment is tagged with  $^{125}\text{I}$ ,  $^{99}\text{Tc}$ ,  $\text{Gd}^{+++}$ , or  $\text{Fe}^{++}$ . Green fluorescent protein is used as a colorimetric tag.

[0012] The invention also includes a soluble fragment of HAAH. The soluble HAAH polypeptide contains an extracellular domain and optionally lacks part or all of the cytoplasmic domain or transmembrane domain of HAAH. In one example, the fragment lacks residues 660-758 of SEQ ID NO:2. In another example, the fragment lacks residues 679-697 (His motif) of SEQ ID NO:2. In yet another example, the fragment, lacks at least one residue of SEQ ID NO:2, the residue being selected from the group consisting of residue 661, 662, 663, 670, 671, 672, and 673. An HAAH fragment is an HAAH polypeptide, the length of which is less than that of a full-length HAAH protein. The full-length HAAH protein is shown in Table 1.

[0013] Diagnostic kits are also encompassed by the invention. For example, a kit for detecting a tumor cell contains an antibody, or fragment thereof, which binds to HAAH. The kit optionally contains a means for detecting binding of the antibody to the tumor cell. For example, the kit contains a detectable marker, e.g., a nonradioactive marker such as

$\text{Gd}^{+++}$  or  $\text{Fe}^{++}$  or a radioactive compound. The kit may also contain instructions for use, a standard reagent for determining positive antibody binding, or a negative control for determining lack of antibody binding. The components are packaged together in a kit.

[0014] The assay format described herein is useful to generate temporal data used for prognosis of malignant disease. A method for prognosis of a malignant neoplasm of a mammal is carried out by (a) contacting a bodily fluid from the mammal with an antibody which binds to an HAAH polypeptide under conditions sufficient to form an antigen-antibody complex and detecting the antigen-antibody complex; (b) quantitating the amount of complex to determine the level of HAAH in the fluid; and (c) comparing the level of HAAH in the fluid with a normal control level of HAAH. An increasing level of HAAH over time indicates a progressive worsening of the disease, and therefore, an adverse prognosis.

[0015] The invention also includes an antibody which binds to HAAH. The antibody preferably binds to a site in the carboxyterminal catalytic domain of HAAH. Alternatively, the antibody binds to an epitope that is exposed on the surface of the cell. The antibody is a polyclonal antiserum or monoclonal antibody. The invention encompasses not only an intact monoclonal antibody, but also an immunologically-active antibody fragment, e.g., a Fab or (Fab)<sub>2</sub> fragment; an engineered single chain Fv molecule; or a chimeric molecule, e.g., an antibody which contains the binding specificity of one antibody, e.g., of murine origin, and the remaining portions of another antibody, e.g., of human origin. Preferably the antibody is a monoclonal antibody such as FB50, 5C7, 5E9, 19B, 48A, 74A, 78A, 86A, HA238A, HA221, HA 239, HA241, HA329, or HA355. Antibodies which bind to the same epitopes as those monoclonal antibodies are also within the invention.

[0016] An HAAH-specific intrabody is a recombinant single chain HAAH-specific antibody that is expressed inside a target cell, e.g., tumor cell. Such an intrabody binds to endogenous intracellular HAAH and inhibits HAAH enzymatic activity or prevents HAAH from binding to an intracellular ligand. HAAH-specific intrabodies inhibit intracellular signal transduction, and as a result, inhibit growth of tumors, which overexpress HAAH.

[0017] A kit for diagnosis of a tumor in a mammal contains an HAAH-specific antibody. The diagnostic assay kit is preferentially formulated in a standard two-antibody binding format in which one HAAH-specific antibody captures HAAH in a patient sample and another HAAH-specific antibody is used to detect captured HAAH. For example, the capture antibody is immobilized on a solid phase, e.g., an assay plate, an assay well, a nitrocellulose membrane, a bead, a dipstick, or a component of an elution column. The second antibody, i.e., the detection antibody, is typically tagged with a detectable label such as a colorimetric agent or radioisotope.

[0018] Also within the invention is a method of inhibiting tumor growth in a mammal, which is carried out by administering to the mammal a compound which inhibits expression or enzymatic activity of HAAH. Preferably, the compound is substantially pure nucleic acid molecule such as an HAAH antisense DNA, the sequence of which is complementary to a coding sequence of HAAH. Expression of

HAAH is inhibited by contacting mammalian cells, e.g., tumor cells, with HAAH antisense DNA or RNA, e.g., a synthetic HAAH antisense oligonucleotide. The sequence of the antisense is complementary to a coding or noncoding region of a HAAH gene. For example, the sequence is complementary to a nucleotide sequence in the 5' untranslated region of a HAAH gene. Examples of HAAH antisense oligonucleotides which inhibit HAAH expression in mammalian cells include oligonucleotides containing SEQ ID NO:10, 11, or 12. An HAAH antisense nucleic acid is introduced into glioblastoma cells or other tumor cells which overexpress HAAH. Binding of the antisense nucleic acid to an HAAH transcript in the target cell results in a reduction in HAAH production by the cell. By the term "antisense nucleic acid" is meant a nucleic acid (RNA or DNA) which is complementary to a portion of an mRNA, and which hybridizes to and prevents translation of the mRNA. Preferably, the antisense DNA is complementary to the 5' regulatory sequence or the 5' portion of the coding sequence of HAAH mRNA (e.g., a sequence encoding a signal peptide or a sequence within exon 1 of the HAAH gene). Standard techniques of introducing antisense DNA into the cell may be used, including those in which antisense DNA is a template from which an antisense RNA is transcribed. The method is to treat tumors in which expression of HAAH is upregulated, e.g., as a result of malignant transformation of the cells. The length of the oligonucleotide is at least 10 nucleotides and may be as long as the naturally-occurring HAAH transcript. Preferably, the length is between 10 and 50 nucleotides, inclusive. More preferably, the length is between 10 and 20 nucleotides, inclusive.

[0019] By "substantially pure DNA or RNA" is meant that the nucleic acid is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank a HAAH gene. The term therefore includes, for example, a recombinant nucleic acid which is incorporated into a vector, into an autonomously

replicating plasmid or virus, or into the genomic DNA of a procaryote or eucaryote at a site other than its natural site; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant nucleic acid which is part of a hybrid gene encoding additional polypeptide sequence such as a nucleic acid encoding an chimeric polypeptide, e.g., one encoding an antibody fragment linked to a cytotoxic polypeptide. Alternatively, HAAH expression is inhibited by administering a ribozyme or a compound which inhibits binding of Fos or Jun to an HAAH promoter sequence.

[0020] Compounds, which inhibit an enzymatic activity of HAAH, are useful to inhibit tumor growth in a mammal. By enzymatic activity of HAAH is meant hydroxylation of an epidermal growth factor (EGF)-like domain of a polypeptide. For example an EGF-like domain has the consensus sequence  $CX_7CX_4CX_{10}CXCX_8C$  (SEQ ID NO:1). HAAH hydroxylase activity is inhibited intracellularly. For example, a dominant negative mutant of HAAH (or a nucleic acid encoding such a mutant) is administered. The dominant negative HAAH mutant contains a mutation which changes a ferrous iron binding site from histidine of a naturally-occurring HAAH sequence to a non-iron-binding amino acid, thereby abolishing the hydroxylase activity of HAAH. The histidine to be mutated, e.g., deleted or substituted, is located in the carboxyterminal catalytic domain of HAAH. For example, the mutation is located between amino acids 650-700 (such as the His motif, underlined sequence of SEQ ID NO:2) the native HAAH sequence. For example, the mutation is at residues 671, 675, 679, or 690 of SEQ ID NO:2. An HAAH-specific intrabody is also useful to bind to HAAH and inhibit intracellular HAAH enzymatic activity, e.g., by binding to an epitope in the catalytic domain of HAAH. Other compounds such as L-mimosine or hydroxypyridone are administered directly into a tumor site or systemically to inhibit HAAH hydroxylase activity.

TABLE 1

| Amino acid sequence of HAAH                                       |     |
|-------------------------------------------------------------------|-----|
| MAQRKNAKSS GNSSSSGSGS GSTSAGSSSP GARRETKHGG HKNGRKGGLS GTSFFTWFMV | 61  |
| IALLGVWTSV AVVWFDLVDY EEVLGKLGIIY DADGDGDFDV DDAKVLGLK ERSTSEPAVP | 121 |
| PEEAEPHTEP EEQVPVEAEP QNIEDEAKEQ IQSLLHEMVH AEHVEGEDLQ QEDGPTGEPQ | 181 |
| QEDDEFMAT DVDDRFFETLE PEVSHEETEH SYHVEETVSQ DCNQDMEEMM SEQENPDSSE | 241 |
| PVVEDERLHH DTDVVTYQVY EEQAVYEPL ENEGIEITEVT APPEDNPVED SQVIVEEVSI | 301 |
| FPVEEQQVEP PETNRKTDDE EQKAKVKKKK PKLLNKFDKT IKAELDAAEK LRKRGKIEEA | 361 |
| VNAFKELVRK YPQSPRARYG KAQCEDDLAE KRRSNEVLRG AIETYQEVAS LPDVPADLLK | 421 |
| LSLKRRSDRQ QFLGHMRGSL LTLQRLVQLF PNDTSLKNDL GVGYLLIGDN DNAKKVYEEV | 481 |
| LSVTPNDGFA KVHYGFILKA QNKIAESIPY LKEGIESGDP GTDDGRFYFH LGDAMQVRGN | 541 |
| KEAYKWYELG HKRGHFASVW QRSLYNVNGL KAQPWWTPKE TGYTELVKSL ERNWKILRDE | 601 |
| GLAVMDKAKG LFLPEDENLR EKGDSQFTL WQQGRRNENA CKGAPKTCTL LEKFPETTGC  | 661 |

TABLE 1-continued

| Amino acid sequence of HAAH                                                                            |
|--------------------------------------------------------------------------------------------------------|
| RRGQIKYSIM <b>HPG</b> THVWPHT <u>GPTN</u> CRLRMH <u>LGLVIP</u> KEGC KIRCANE <b>T</b> RT WEEGKVLIFD 721 |
| DSFEHEVWQD ASSFRLIFIV DVWHPELTPQ QRRSLPAI                                                              |

(SEQ ID NO: 2; GENBANK Accession No. S83325; His motif is underlined; conserved sequences within the catalytic domain are designated by bold type)

[0021] For example, a compound which inhibits HAAH hydroxylation is a polypeptide that binds a HAAH ligand but does not transduce an intracellular signal or a polypeptide which contains a mutation in the catalytic site of HAAH. Such a polypeptide contains an amino acid sequence that is at least 50% identical to a naturally-occurring HAAH amino acid sequence or a fragment thereof and which has the ability to inhibit HAAH hydroxylation of substrates containing an EGF-like repeat sequence. More preferably, the polypeptide contains an amino acid sequence that is at least 75%, more preferably at least 85%, more preferably at least 95% identical to SEQ ID NO:2.

[0022] A substantially pure HAAH polypeptide or HAAH-derived polypeptide such as a mutated HAAH polypeptide is preferably obtained by expression of a recombinant nucleic acid encoding the polypeptide or by chemically synthesizing the protein. A polypeptide or protein is

substantially pure when it is separated from those contaminants which accompany it in its natural state (proteins and other naturally-occurring organic molecules). Typically, the polypeptide is substantially pure when it constitutes at least 60%, by weight, of the protein in the preparation. Preferably, the protein in the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, HAAH. Purity is measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. Accordingly, substantially pure polypeptides include recombinant polypeptides derived from a eucaryote but produced in *E. coli* or another procaryote, or in a eucaryote other than that from which the polypeptide was originally derived.

[0023] Nucleic acid molecules which encode such HAAH or HAAH-derived polypeptides are also within the invention.

TABLE 2

| HAAH cDNA sequence                                                           |  |
|------------------------------------------------------------------------------|--|
| cggaccgtgc <u>aatgg</u> cccag cgtaagaatg ccaagagcag cggcaacagc agcagcagcg 61 |  |
| gctccggcag cggtagcacg agtgcgggca gcagcagccc cggggcccgag agagagacaa 121       |  |
| agcatggagg acacaagaat gggaggaaag gcggactctc gggaacttca ttcttcacgt 181        |  |
| ggtttatggt gattgcattg ctgggcgtct ggacatctgt agctgtcgtt tggtttgatc 241        |  |
| ttgttgacta tgaggaaagt ctaggaaaac taggaatcta tgatgctgat ggtgatggag 301        |  |
| atthttgatgt ggatgatgcc aaagttttat taggacttaa agagagatct acttcagagc 361       |  |
| cagcagtcac gccagaagag gctgagccac aactgagcc cgaggagcag gttcctgtgg 421         |  |
| aggcagaacc ccagaatatc gaagatgaag caaaagaaca aattcagtc cttctccatg 481         |  |
| aaatggtaca cgcagaacat gttgagggag aagacttgca acaagaagat ggaccacag 541         |  |
| gagaaccaca acaagaggat gatgagtttc ttatggcgac tgatgtagat gatagatttg 601        |  |
| agaccctgga acctgaagta tctcatgaag aaaccgagca tagttaccac gtggaagaga 661        |  |
| cagtttcaca agactgtaat caggatatgg aagagatgat gtctgagcag gaaaatccag 721        |  |
| attccagtga accagtagta gaagatgaaa gattgcacca tgatacagat gatgtaacat 781        |  |
| accaagtcta tgaggaaaca gcagtatatg aacctctaga aaatgaaggg atagaaatca 841        |  |
| cagaagtaac tgctccccct gaggataatc ctgtagaaga ttcacaggtta attgtagaag 901       |  |
| aagtaagcat ttttctctgt gaagaacagc aggaagtacc accagaaaca aatagaaaaa 961        |  |
| cagatgatcc agaacaaaaa gcaaaagtta agaaaaagaa gcctaaactt ttaaataaat 1021       |  |
| ttgataagac tattaagact gaacttgatg ctgcagaaaa actccgtaaa aggggaaaaa 1081       |  |

TABLE 2-continued

| HAAH cDNA sequence                                                 |      |
|--------------------------------------------------------------------|------|
| ttgaggaagc agtgaatgca tttaagaac tagtacgcaa ataccctcag agtccacgag   | 1141 |
| caagatatgg gaaggcgagc tgtgaggatg atttggctga gaagaggaga agtaatgagg  | 1201 |
| tgcctacgtgg agccatcgag acctaccaag aggtggccag cctacctgat gtccctgcag | 1261 |
| acctgctgaa gctgagtttg aagcgtcgct cagacaggca acaatttcta ggtcatatga  | 1321 |
| gagggtccct gcttaccctg cagagattag ttcaactatt tcccaatgat acttccttaa  | 1381 |
| aaaaagacct tggcgtggga tacctcttga taggagataa tgacaatgca aagaaagttt  | 1441 |
| atgaagaggt gctgagtgtg acacctaatt atggctttgc taaagtccat tatggcttca  | 1501 |
| tcctgaaggc acagaacaaa attgtgaga gcacccata tttaaaggaa ggaatagaat    | 1561 |
| ccggagatcc tggcactgat gatgggagat tttatttcca cctgggggat gccatgcaga  | 1621 |
| gggttgggaa caaagaggca tataagtggg atgagcttgg gcacaagaga ggacactttg  | 1681 |
| catctgtctg gcaacgctca ctctacaatg tgaatggact gaaagcacag ccttggtgga  | 1741 |
| ccccaaaaga aacgggctac acagagttag taaagtcttt agaaagaaac tggaggttaa  | 1801 |
| tccgagatga aggccttgca gtgatggata aagccaaagg tctcttcctg cctgaggatg  | 1861 |
| aaaacctgag ggaanaaggg gactggagcc agttcacgct gtggcagcaa ggaagaagaa  | 1921 |
| atgaaaatgc ctgcaaggga gctcctaaaa cctgtacctt actagaaaag tccccgaga   | 1981 |
| caacaggatg cagaagagga cagatcaaat attccatcat gcaccccgagg actcacgtgt | 2041 |
| ggcgcacac agggccaca aactgcaggc tccgaatgca cctgggcttg gtgattccca    | 2101 |
| aggaaggctg caagattcga tgtgccaacg agaccaggac ctgggaggaa ggcaagggtg  | 2161 |
| tcactcttga tgactccttt gagcacgagg tatggcagga tgcctcatct ttccggtga   | 2221 |
| tattcatcgt ggatgtgtg catccggaac tgacaccaca gcagagacgc agccttcag    | 2281 |
| caatttagca tgaattcatg caagcttggg aaactctgga gaga                   |      |

(SEQ ID NO: 3; GENBANK Accession No. S83325; codon encoding initiating methionine is underlined).

[0024] Methods of inhibiting tumor growth also include administering a compound which inhibits HAAH hydroxylation of a NOTCH polypeptide. For example, the compound inhibits hydroxylation of an EGF-like cysteine-rich repeat sequence in a NOTCH polypeptide, e.g., one containing the consensus sequence CDXXXCXXKXGNGX-CDXXCNNAACXXDGXDC (SEQ ID NO:4). Polypeptides containing an EGF-like cysteine-rich repeat sequence are administered to block hydroxylation of endogenous NOTCH.

[0025] Growth of a tumor which overexpresses HAAH is also inhibited by administering a compound which inhibits signal transduction through the insulin receptor substrate (IRS) signal transduction pathway. Preferably the compound inhibits IRS phosphorylation. For example, the compound is a peptide or non-peptide compound which binds to and inhibits phosphorylation at residues 46, 465, 551, 612, 632, 662, 732, 941, 989, or 1012 of SEQ ID NO:5. Compounds include polypeptides such those which block an IRS phosphorylation site such as a Glu/Tyr site. Antibodies such as those which bind to a carboxyterminal domain of IRS containing a phosphorylation site block IRS phosphorylation, and as a consequence, signal transduction along the

pathway. Inhibition of IRS phosphorylation in turn leads to inhibition of cell proliferation. Other compounds which inhibit IRS phosphorylation include vitamin D analogue EB1089 and Wortmannin.

[0026] HAAH-overproducing tumor cells were shown to express HAAH both intracellularly and on the surface of the tumor cell. Accordingly, a method of killing a tumor cell is carried out by contacting such a tumor cell with a cytotoxic agent linked to an HAAH-specific antibody. The HAAH-specific antibody (antibody fragment, or ligand which binds to extracellular HAAH) directs the chimeric polypeptide to the surface of the tumor cell allowing the cytotoxic agent to damage or kill the tumor cell to which the antibody is bound. The monoclonal antibody binds to an epitope of HAAH such as an epitope exposed on the surface of the cell or in the catalytic site of HAAH. The cytotoxic composition preferentially kills tumor cells compared to non-tumor cell.

[0027] Screening methods to identify anti-tumor agents which inhibit the growth of tumors which overexpress HAAH are also within the invention. A screening method used to determine whether a candidate compound inhibits HAAH enzymatic activity includes the following steps: (a) providing a HAAH polypeptide, e.g., a polypeptide which

contains the carboxyterminal catalytic site of HAAH; (b) providing a polypeptide comprising an EGF-like domain; (c) contacting the HAAH polypeptide or the EGF-like polypeptide with the candidate compound; and (d) determining hydroxylation of the EGF-like polypeptide of step (b). A decrease in hydroxylation in the presence of the candidate compound compared to that in the absence of the compound indicates that the compound inhibits HAAH hydroxylation of EGF-like domains in proteins such as NOTCH.

**[0028]** Anti-tumor agents which inhibit HAAH activation of NOTCH are identified by (a) providing a cell expressing HAAH; (b) contacting the cell with a candidate compound; and (c) measuring translocation of activated NOTCH to the nucleus of the cell. Translocation is measured by using a reagent such as an antibody which binds to a 110 kDa activation fragment of NOTCH. A decrease in translocation in the presence of the candidate compound compared to that in the absence of the compound indicates that the compound inhibits HAAH activation of NOTCH, thereby inhibiting NOTCH-mediated signal transduction and proliferation of HAAH-overexpressing tumor cells.

**[0029]** Nucleotide and amino acid comparisons described herein were carried out using the Lasergene software package (DNASTAR, Inc., Madison, Wis.). The MegAlign module used was the Clustal V method (Higgins et al., 1989, CABIOS 5(2):151-153). The parameter used were gap penalty 10, gap length penalty 10.

**[0030]** Hybridization is carried out using standard techniques, such as those described in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, 1989). "High stringency" refers to nucleic acid hybridization and wash conditions characterized by high temperature and low salt concentration, e.g., wash conditions of 65° C. at a salt concentration of 0.1×SSC. "Low" to "moderate" stringency refers to DNA hybridization and wash conditions characterized by low temperature and high salt concentration, e.g., wash conditions of less than 60° C. at a salt concentration of 1.0×SSC. For example, high stringency conditions include hybridization at 42° C. in the presence of 50% formamide; a first wash at 65° C. in the presence of 2×SSC and 1% SDS; followed by a second wash at 65° C. in the presence of 0.1×SSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to an HAAH gene sequence are detected by, for example, hybridization at about 42° C. in the absence of formamide; a first wash at 42° C., 6×SSC, and 1% SDS; and a second wash at 50° C., 6×SSC, and 1% SDS.

**[0031]** Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**[0032]** FIG. 1 is a bar graph showing colony formation induced by transient transfection of NIH-3T3 cells with various aspartyl (asparaginyl) beta-hydroxylase (AAH) cDNAs. Colony formation was induced by transient transfection with 10 µg DNA. In contrast, the mutant murine AAH construct without enzymatic activity has no transforming activity. The data is presented as mean number of transformed foci±SEM.

**[0033]** FIG. 2 is a bar graph showing the results of a densitometric analysis of a Western blot assay of proteins

produced by various murine AAH stably transfected cell clones. In clones 7 and 18, there was a modest increase in HAAH gene expression, while the overexpression was to a lesser degree in clone 16.

**[0034]** FIGS. 3A-B are bar graphs showing colony formation in soft agar exhibited by HAAH stably transfected clones compared to HAAH enzymatic activity. FIG. 3A shows a measurement of murine AAH enzymatic activity in clones 7, 16 and 18, and FIG. 3B shows colony formation exhibited by clones 7, 16 and 18. Data is presented as mean number of colonies 10 days after plating±SEM. All three clones with modest increases in HAAH enzymatic activity, that correlated with protein expression, exhibited anchorage independent growth.

**[0035]** FIG. 4 is a bar graph showing tumor formation in nude mice injected with transfected clones overexpressing murine AAH. Tumor growth was assessed after 30 days. Mean tumor weight observed in mice injected with clones 7, 16 and 18 as compared to mock DNA transfected clone. All animals, which were injected with clones overexpressing HAAH, developed tumors.

**[0036]** FIGS. 5A-D are bar graphs showing increased AAH expression in PNET2 (FIG. 5A, 5C) and SH-Sy5y (FIG. 5B) cells treated with retinoic acid (FIGS. 5A, 5B) or phorbol ester myristate (PMA; FIG. 5C) to induce neurite outgrowth as occurs during tumor cell invasion. The cells were treated with 10 µM retinoic acid or 100 nM PMA for 0, 1, 2, 3, 4, or 7 days. Cell lysates were analyzed by Western blot analysis using an HAAH-specific monoclonal antibody to detect the 85 kDa AAH protein. The levels of immunoreactivity were measured by volume densitometry (arbitrary units). The graphs indicate the mean±S.D. of results obtained from three separate experiments. In FIG. 5D, PNET2 cells were treated for 24 hours with sub-lethal concentrations of H<sub>2</sub>O<sub>2</sub> to induce neurite retraction. Viability of greater than 90% of the cells was demonstrated by Trypan blue dye exclusion. Similar results were obtained for SH-Sy5y cells.

**[0037]** FIG. 6 is a bar graph showing the effects of AAH over-expression on the levels of anti-apoptosis (Bcl-2), cell cycle-mitotic inhibitor (p16 and p21/Waf1), and proliferation (proliferating cell nuclear antigen; PCNA) molecules. PNET2 neuronal cells were stably transfected with the full-length human cDNA encoding AAH (pHAAH) or empty vector (pcDNA). AAH gene expression was under control of a CMV promoter. Western blot analysis was performed with cell lysates prepared from cultures that were 70 to 80 percent confluent. Protein loading was equivalent in each lane. Replicate blots were probed with the different antibodies. Bar graphs depict the mean S.D.'s of protein expression levels measured in three experiments. All differences are statistically significant by Student T-test analysis (P<0.01-P<0.001).

**[0038]** FIG. 7 is a diagram of showing the components of the IRS-1 signal transduction pathway.

**[0039]** FIG. 8 is a line graph showing growth curves generated in cells expressing the antisense HAAH compared to controls expressing GFP.

**[0040]** FIG. 9 is a diagram of the functional domains of the hIRS-1 protein and structural organization of the point mutants. All mutant and "wild type" hIRS-1 proteins con-

struct contain a FLAG (F) epitope (DYKDDDDK; SEQ ID NO:7) at the C-terminus. PH and PTB indicate pleckstrin homology and phosphotyrosine binding, regions, respectively.

#### DETAILED DESCRIPTION

[0041] HAAH is a protein belonging to the alpha-ketoglutarate dependent dioxygenase family of prolyl and lysyl hydroxylases which play a key role in collagen biosynthesis. This molecule hydroxylates aspartic acid or asparagine residues in EGF-like domains of several proteins in the presence of ferrous iron. These EGF-like domains contain conserved motifs, that form repetitive sequences in proteins such as clotting factors, extracellular matrix proteins, LDL receptor, NOTCH homologues, or NOTCH ligand homologues.

[0042] The alpha-ketoglutarate-dependent dioxygenase aspartyl (asparaginyl) beta-hydroxylase (AAH) specifically hydroxylates one aspartic or asparagine residue in EGF-like domains of various proteins. The 4.3-kb cDNA encoding the human AspH (hAspH) hybridizes with 2.6 kb and 4.3 kb transcripts in transformed cells, and the deduced amino acid sequence of the larger transcript encodes an protein of about 85 kDa. Both in vitro transcription and translation and Western blot analysis also demonstrate a 56-kDa protein that may result from posttranslational cleavage of the catalytic C-terminus.

[0043] A physiological function of AAH is the post-translational beta-hydroxylation of aspartic acid in vitamin K-dependent coagulation proteins. However, the abundant expression of AAH in several malignant neoplasms, and low levels of AAH in many normal cells indicate a role for this enzyme in malignancy. The AAH gene is also highly expressed in cytotrophoblasts, but not syncytiotrophoblasts of the placenta. Cytotrophoblasts are invasive cells that mediate placental implantation. The increased levels of AAH expression in human cholangiocarcinomas, hepatocellular carcinomas, colon cancers, and breast carcinomas were primarily associated with invasive or metastatic lesions. Moreover, overexpression of AAH does not strictly reflect increased DNA synthesis and cellular proliferation since high levels of AAH immunoreactivity were observed in 100 percent of cholangiocarcinomas, but not in human or experimental disease processes associated with regeneration or nonneoplastic proliferation of bile ducts. AAH overexpression and attendant high levels of beta hydroxylase activity lead to invasive growth of transformed neoplastic cells. Detection of an increase in HAAH expression is useful for early and reliable diagnosis of the cancer types which have now been characterized as overexpressing this gene product.

#### Diagnosis of Malignant Tumors

[0044] HAAH is overexpressed in many tumors of endodermal origin and in at least 95% of CNS tumors compared to normal noncancerous cells. An increase in HAAH gene product in a patient-derived tissue sample (e.g., solid tissue or bodily fluid) is carried out using standard methods, e.g., by Western blot assays or a quantitative assay such as ELISA. For example, a standard competitive ELISA format using an HAAH-specific antibody is used to quantify patient HAAH levels. Alternatively, a sandwich ELISA using a first antibody as the capture antibody and a second HAAH-specific antibody as a detection antibody is used.

[0045] Methods of detecting HAAH include contacting a component of a bodily fluid with an HAAH-specific antibody bound to solid matrix, e.g., microtiter plate, bead, dipstick. For example, the solid matrix is dipped into a patient-derived sample of a bodily fluid, washed, and the solid matrix is contacted with a reagent to detect the presence of immune complexes present on the solid matrix.

[0046] Proteins in a test sample are immobilized on (e.g., bound to) a solid matrix. Methods and means for covalently or noncovalently binding proteins to solid matrices are known in the art. The nature of the solid surface may vary depending upon the assay format. For assays carried out in microtiter wells, the solid surface is the wall of the microtiter well or cup. For assays using beads, the solid surface is the surface of the bead. In assays using a dipstick (i.e., a solid body made from a porous or fibrous material such as fabric or paper) the surface is the surface of the material from which the dipstick is made. Examples of useful solid supports include nitrocellulose (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates), polyvinylidene fluoride (known as IMMULON™), diazotized paper, nylon membranes, activated beads, and Protein A beads. The solid support containing the antibody is typically washed after contacting it with the test sample, and prior to detection of bound immune complexes. Incubation of the antibody with the test sample is followed by detection of immune complexes by a detectable label. For example, the label is enzymatic, fluorescent, chemiluminescent, radioactive, or a dye. Assays which amplify the signals from the immune complex are also known in the art, e.g., assays which utilize biotin and avidin.

[0047] An HAAH-detection reagent, e.g., an antibody, is packaged in the form of a kit, which contains one or more HAAH-specific antibodies, control formulations (positive and/or negative), and/or a detectable label. The assay may be in the form of a standard two-antibody sandwich assay format known in the art.

#### Production of HAAH-specific Antibodies

[0048] Anti-HAAH antibodies were obtained by techniques well known in the art. Such antibodies are polyclonal or monoclonal. Polyclonal antibodies were obtained using standard methods, e.g., by the methods described in Ghose et al., *Methods in Enzymology*, Vol. 93, 326-327, 1983. An HAAH polypeptide, or an antigenic fragment thereof, was used as the immunogen to stimulate the production of polyclonal antibodies in the antisera of rabbits, goats, sheep, or rodents. Antigenic polypeptides for production of both polyclonal and monoclonal antibodies useful as immunogens include polypeptides which contain an HAAH catalytic domain. For example, the immunogenic polypeptide is the full-length mature HAAH protein or an HAAH fragment containing the carboxyterminal catalytic domain e.g., an HAAH polypeptide containing the His motif of SEQ ID NO:2.

[0049] Antibodies which bind to the same epitopes as those antibodies disclosed herein are identified using standard methods, e.g., competitive binding assays, known in the art.

[0050] Monoclonal antibodies were obtained by standard techniques. Ten  $\mu$ g of purified recombinant HAAH polypep-

tide was administered to mice intraperitoneally in complete Freund's adjuvant, followed by a single boost intravenously (into the tail vein) 3-5 months after the initial inoculation. Antibody-producing hybridomas were made using standard methods. To identify those hybridomas producing antibodies that were highly specific for an HAAH polypeptide, hybridomas were screened using the same polypeptide immunogen used to immunize. Those antibodies which were identified as having HAAH-binding activity are also screened for the ability to inhibit HAAH catalytic activity using the enzymatic assays described below. Preferably, the antibody has a binding affinity of at least about  $10^8$  liters/mole and more preferably, an affinity of at least about  $10^9$  liters/mole.

[0051] Monoclonal antibodies are humanized by methods known in the art, e.g., MAbs with a desired binding specificity can be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, Calif.).

[0052] HAAH-specific intrabodies are produced as follows. Following identification of a hybridoma producing a suitable monoclonal antibody, DNA encoding the antibody is cloned. DNA encoding a single chain HAAH-specific antibody in which heavy and light chain variable domains are separated by a flexible linker peptide is cloned into an expression vector using known methods (e.g., Marasco et al., 1993, Proc. Natl. Acad. Sci. USA 90:7889-7893 and Marasco et al., 1997, Gene Therapy 4:11-15). Such constructs are introduced into cells, e.g., using standard gene delivery techniques for intracellular production of the antibodies. Intracellular antibodies, i.e., intrabodies, are used to inhibit signal transduction by HAAH. Intrabodies which bind to a carboxyterminal catalytic domain of HAAH inhibit the ability of HAAH to hydroxylate EGF-like target sequences.

[0053] Methods of linking HAAH-specific antibodies (or fragments thereof) which bind to cell surface exposed epitopes of HAAH on the surface of a tumor cell are linked to known cytotoxic agents, e.g., ricin or diphtheria toxin, using known methods.

#### Deposit of Biological Materials

[0054] Under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, hybridoma FB501 (which produces monoclonal antibody FB50), hybridoma HA386A (which produces monoclonal antibody 86A), hybridoma HA15C7A (which produces monoclonal antibody 5C7), and hybridoma HA219B (which produces monoclonal antibody 19B) were deposited on May 17, 2001, with the American Type Culture Collection (ATCC) of 10801 University Boulevard, Manassas, Va. 20110-2209 USA.

[0055] Applicants' assignee represents that the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request

for the furnishing of a sample of the deposited plasmid, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicant's assignee acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

#### Methods of Treating Malignant Tumors

[0056] Patients with tumors characterized as overexpressing HAAH as such tumors of endodermal origin or CNS tumors are treated by administering HAAH antisense nucleic acids.

[0057] Antisense therapy is used to inhibit expression of HAAH in patients suffering from hepatocellular carcinomas, cholangiocarcinomas, glioblastomas and neuroblastomas. For example, an HAAH antisense strand (either RNA or DNA) is directly introduced into the cells in a form that is capable of binding to the mRNA transcripts. Alternatively, a vector containing a sequence which, which once within the target cells, is transcribed into the appropriate antisense mRNA, may be administered. Antisense nucleic acids which hybridize to target mRNA decrease or inhibit production of the polypeptide product encoded by a gene by associating with the normally single-stranded mRNA transcript, thereby interfering with translation and thus, expression of the protein. For example, DNA containing a promoter, e.g., a tissue-specific or tumor specific promoter, is operably linked to a DNA sequence (an antisense template), which is transcribed into an antisense RNA. By "operably linked" is meant that a coding sequence and a regulatory sequence(s) (i.e., a promoter) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

[0058] Oligonucleotides complementary to various portions of HAAH mRNA were tested in vitro for their ability to decrease production of HAAH in tumor cells (e.g., using the FOCUS hepatocellular carcinoma (HCC) cell line) according to standard methods. A reduction in HAAH gene product in cells contacted with the candidate antisense composition compared to cells cultured in the absence of the candidate composition is detected using HAAH-specific antibodies or other detection strategies. Sequences which decrease production of HAAH in in vitro cell-based or cell-free assays are then be tested in vivo in rats or mice to confirm decreased HAAH production in animals with malignant neoplasms.

[0059] Antisense therapy is carried out by administering to a patient an antisense nucleic acid by standard vectors and/or gene delivery systems. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses and adeno-associated viruses, among others. A reduction in HAAH production results in a decrease in signal transduction via the IRS signal transduction pathway. A therapeutic nucleic acid composition is formulated in a pharmaceutically acceptable carrier. The therapeutic composition may also include a gene delivery system as described above. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal: e.g., physiological

saline. A therapeutically effective amount of a compound is an amount which is capable of producing a medically desirable result such as reduced production of an HAAH gene product or a reduction in tumor growth in a treated animal.

[0060] Parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal delivery routes, may be used to deliver nucleic acids or HAAH-inhibitory peptides or non-peptide compounds. For treatment of CNS tumors, direct infusion into cerebrospinal fluid is useful. The blood-brain barrier may be compromised in cancer patients, allowing systemically administered drugs to pass through the barrier into the CNS. Liposome formulations of therapeutic compounds may also facilitate passage across the blood-brain barrier.

[0061] Dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular nucleic acid to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosage for intravenous administration of nucleic acids is from approximately  $10^6$  to  $10^{22}$  copies of the nucleic acid molecule.

[0062] Ribozyme therapy is also be used to inhibit HAAH gene expression in cancer patients. Ribozymes bind to specific mRNA and then cut it at a predetermined cleavage point, thereby destroying the transcript. These RNA molecules are used to inhibit expression of the HAAH gene according to methods known in the art (Sullivan et al., 1994, J. Invest. Derm. 103:85S-89S; Czubyko et al., 1994, J. Biol. Chem. 269:21358-21363; Mahieu et al., 1994, Blood 84:3758-65; Kobayashi et al. 1994, Cancer Res. 54:1271-1275).

#### HAAH-specific Antibodies Inhibit Tumor Cell Growth

[0063] HAAH-specific antibodies inhibit the proliferation of tumor cells in culture. Two different HAAH-specific antibodies, FB-50 and 5C7, were tested. Tumor cells (a hepatocarcinoma cell line, a lung carcinoma cell line, and a breast carcinoma cell line) were seeded in a 96 well plate and incubated with varying concentrations of antibody for 48 hours. The cells were fixed with acetone. Cell growth was monitored using a sulforhodamine B dye binding assay. The data indicated a reduction in cell viability and proliferation in the presence of FB50 compared to in its absence.

#### Passive Immunization

[0064] The HAAH-specific antibodies described herein are used to inhibit the growth of a tumor cell or kill the tumor cell.

[0065] Purified antibody preparations (e.g., a purified monoclonal antibody, an antibody fragment, or single chain antibody) is administered to an individual diagnosed with a tumor or at risk of developing a tumor. The antibody preparations are administered using methods known in the art of passive immunization, e.g., intravenously or intramuscularly. The antibodies used in the methods described herein are formulated in a physiologically-acceptable excipient. Such excipients, e.g., physiological saline, are known in the art.

[0066] The antibody is preferably a high-affinity antibody, e.g., an IgG-class antibody or fragment or single chain

thereof. Alternatively, the antibody is an IgM isotype. Antibodies are monoclonal, e.g., a murine monoclonal antibody or fragment thereof, or a murine monoclonal antibody, which has been humanized. The antibody is a human monoclonal antibody. The affinity of a given monoclonal antibody is further increased using known methods, e.g., by selecting for increasingly higher binding capacity (e.g., according to the method described in Boder et al., 2000, Proc. Natl. Acad. Sci. U.S.A. 97:10701-10705). Optionally, the antibody, antibody fragment, or high affinity single chain antibody is conjugated to a toxic moiety prior to administration. Toxic moieties suitable for conjugation include ricin, *Psuedomonas* toxin, Diphtheria toxin as well as radioisotopes and chemotherapeutic agents known in the art. Such antibody toxins damage or kill a tumor cell upon binding to the tumor cell or upon internalization into the cytoplasm of the tumor cell.

[0067] Antibody preparations or antibody-toxin preparations are administered at doses of approximately 0.01-2 mL/kg of body weight. Doses are readministered weekly or monthly as necessary to reduce tumor load in a treated individual.

#### Active Immunization

[0068] Active vaccination is the process of inducing an animal to respond to an antigen. During vaccination, cells, which recognize the antigen (B cells or cytotoxic T cells), are clonally expanded. In addition, the population of helper T cells specific for the antigen also increase. Vaccination also involves specialized antigen presenting cells, which can process the antigen and display it in a form which can stimulate one of the two pathways. Antigen recognition followed by immune cell expansion and activation leads to the production of antigen-specific antibodies and antigen-specific cellular immune responses. Successful immunization is indicated by an increase in the level of HAAH-specific antibody titer in serum of an immunized individual compared to the level prior to immunization. Preferably, the HAAH-specific antibody titer is at least 10%, more preferably at least 50%, more preferably at least 100%, and most preferably 200% greater than the titer prior to immunization.

[0069] An individual is immunized with an AAH (e.g., HAAH) polypeptide or a polynucleotide encoding the peptide. For example, a human patient is immunized with full-length 52 kDa HAAH. Standard adjuvant formulations may be simultaneously administered to enhance immunogenicity of the immunizing polypeptide. Alternatively, shorter polypeptides, e.g., immunogenic fragments of HAAH, are used. For example, a polypeptide contains an extracellular catalytic domain of HAAH (e.g., amino acids 650-700 of SEQ ID NO:2). Other immunogenic fragments of HAAH include a fragment contains a binding site for alpha-ketoglutarate, a fragment that lacks a binding site for alpha-ketoglutarate, one which contains a calcium binding site, and one which lacks a binding site for an EGF-like polypeptide.

#### DNA vaccine

[0070] In addition to standard active vaccination using a peptide antigen, DNA vaccination is used to generate an immune response to HAAH, and in turn to tumor cells, which overexpress HAAH. Although HAAH is overexpressed on malignant cells, an effective immune response is

not made by the patient because tumor cells lack appropriate accessory molecules for antigen presentation. The DNA vaccines described herein result in generation of a humoral as well as cellular immunity specific for HAAH (and cells expressing HAAH on their cell surface). For example, not only is an HAAH-specific antibody produced in the immunized individual, HAAH-specific cytotoxic T cells are generated. HAAH-specific cytotoxic T cells kill tumor cells, thereby reducing tumor load in the immunized individual.

**[0071]** A polynucleotide encoding an AAH polypeptide (full-length or an immunogenic fragment of AAH) is introduced into an individual by known methods, e.g., particle bombardment or direct injection via needle. Typically, the antigen (or DNA encoding the antigen) is delivered intramuscularly. The antigen is also directly injected into other tissues, e.g., tumor sites. DNA is taken up by cells at the point of injection. The cell produces proteins, and the proteins stimulate the immune system of the immunized individual resulting, e.g., in generation of an HAAH-specific antibody. Cellular immunity, e.g., cytotoxic T cells, are also generated.

**[0072]** An effective DNA or mRNA dosage is generally be in the range of from about 0.05 micrograms/kg to about 50 mg/kg, usually about 0.005-5 mg/kg of body weight, e.g., 0.5 to 5 mg/kg. The DNA to be administered is naked (in the absence of transfection-facilitating substances) or complexed with compounds, which enhance cellular uptake of the polynucleotide (e.g., charged lipids, lipid complexes or liposomes). For example, the polynucleotide is administered with Lipofectin™ or precipitating agents such as CaPO<sub>4</sub>. The transfected cells, e.g., non-proliferating muscle cells, produce the recombinant antigenic polypeptide for at least one month and up to several months, e.g. 3-6 months. Alternatively, transitory expression of a polypeptide is achieved by introducing the polynucleotide construct into a tissue (e.g., non-muscular tissue or tumor tissue). In the latter case, cells of the tissue produce the polypeptide for a shorter period of time, e.g., several days (3-5 days and up to about 20 days). The level of protein or polypeptide expression by target cells is sufficient to induce production of HAAH-specific antibodies. The level of antibody production is measured using standard methods, e.g., evaluation of antibody titer in patient serum, before and after immunization.

**[0073]** The polynucleotides are administered by standard methods, such as by injection into the interstitial space of tissues such as muscles or skin, introduction into the circulation or into body cavities or by inhalation or insufflation. Polynucleotides are injected or otherwise delivered to the animal with a pharmaceutically acceptable liquid carrier, e.g., a liquid carrier, which is aqueous or partly aqueous. The polynucleotides are associated with a liposome (e.g., a cationic or anionic liposome). The polynucleotide includes genetic information necessary for expression by a target cell, such as a promoters.

**[0074]** One advantage of DNA vaccination is that DNA vaccines can result in longer lasting production of the antigenic protein, thereby booster shots reducing or avoiding booster immunizations.

**[0075]** In addition to inducing an immune response, e.g., an HAAH-specific antibody response, by vaccinating with DNA encoding an HAAH polypeptide, a polynucleotide

encoding the antibody itself is introduced. An isolated polynucleotide encoding an HAAH-specific antibody, e.g., variable regions of the antibody, is introduced for production of the antibody in situ. The antibody in turn exerts a therapeutic effect at the target site by binding a cell surface antigen, e.g., extracellular HAAH, or by binding to a catalytic domain of HAAH, to inhibit HAAH function.

#### In Vivo Diagnostic Imaging

**[0076]** The antibodies (antibody fragments, and single chain antibodies) described herein are useful to diagnose the presence of a tumor in tissues as well as bodily fluids. HAAH-specific antibodies are tagged with a detectable label such as a radioisotope or colorimetric agent. The labeled antibody is administered to an individual at risk of developing cancer or an individual who has previously been diagnosed with cancer. For example, the antibodies are useful to diagnose metastases of a tumor, which has been surgically excised or treated by chemotherapeutic or radiotherapeutic methods. The sensitivity of the method is sufficient to detect micrometastases in tissues such as lymph nodes. Early and sensitive diagnosis of tumors in this manner allows prompt therapeutic intervention.

**[0077]** The labeled antibody is administered to an individual using known methods, e.g., intravenously, or direct injection into solid or soft tissues. The antibody is allowed to distribute throughout the tissue or throughout the body for a period of approximately 1 hour to 72 hours. The whole body of the individual is then imaged using methods known in the art. Alternatively, a small portion of the body, e.g., a tissue site suspected of harboring a tumor, is imaged. An increase in antibody binding, as measured by an increase in detection of the label, over the level of baseline binding (to normal tissue) indicates the presence of a tumor at the site of binding.

#### Activation of NOTCH Signaling

**[0078]** NOTCH signalling is activated in cells highly expressing AAH. Overexpression of enzymatically active AAH is shown by a display of the 100 kDa cleaved, active NOTCH-1 (Lane 1, Mock DNA transfected clone; Lane 2, clones 7; and Lane 3, clone 18). In contrast, NOTCH-2 was not activated. There was enhanced expression of the full length Jagged ligand in clones expressing AAH as compared to the mock DNA transfected clone. Tubulin was used as internal control for protein loading.

**[0079]** Only AAH-expressing clones activate Notch expression as a transcription factor and subsequently unregulates Hes-1 gene expression as revealed by competitive RT-PCR. RT-PCR product of GAPDH that served as internal control. Tubulin was used as an internal control for protein loading.

#### Methods of Identifying Compounds that Inhibit HAAH Enzymatic Activity

**[0080]** Aspartyl (asparaginy) beta-hydroxylase (AAH) activity is measured in vitro or in vivo. For example, HAAH catalyzes posttranslational modification of  $\beta$  carbon of aspartyl and asparaginy residues of EGF-like polypeptide domains. An assay to identify compounds which inhibit hydroxylase activity is carried out by comparing the level of hydroxylation in an enzymatic reaction in

which the candidate compound is present compared to a parallel reaction in the absence of the compound (or a predetermined control value). Standard hydroxylase assays carried out in a testtube are known in the art, e.g., Lavaisiere et al., 1996, *J. Clin. Invest.* 98:1313-1323; Jia et al., 1992, *J. Biol. Chem.* 267:14322-14327; Wang et al., 1991, *J. Biol. Chem.* 266:14004-14010; or Gronke et al., 1990, *J. Biol. Chem.* 265:8558-8565. Hydroxylase activity is also measured using carbon dioxide ( $^{14}\text{CO}_2$  capture assay) in a 96-well microtiter plate format (Zhang et al., 1999, *Anal. Biochem.* 271:137-142). These assays are readily automated and suitable for high throughput screening of candidate compounds to identify those with hydroxylase inhibitory activity.

[0081] Candidate compound which inhibit HAAH activation of NOTCH are identified by detecting a reduction in activated NOTCH in a cell which expresses or overexpresses HAAH, e.g., FOCUS HCC cells. The cells are cultured in the presence of a candidate compound. Parallel cultures are incubated in the absence of the candidate compound. To evaluate whether the compound inhibits HAAH activation of NOTCH, translocation of activated NOTCH to the nucleus of the cell is measured. Translocation is measured by detecting a 110 kDa activation fragment of NOTCH in the nucleus of the cell. The activation fragment is cleaved from the large (approximately 300 kDa) transmembrane NOTCH protein upon activation. Methods of measuring NOTCH translocation are known, e.g., those described by Song et al., 1999, *Proc. Natl. Acad. Sci. U.S.A.* 96:6959-6963 or Capobianco et al., 1997, *Mol. Cell Biol.* 17:6265-6273. A decrease in translocation in the presence of the candidate compound compared to that in the absence of the compound indicates that the compound inhibits HAAH activation of NOTCH, thereby inhibiting NOTCH-mediated signal transduction and proliferation of HAAH-overexpressing tumor cells.

[0082] Methods of screening for compounds which inhibit phosphorylation of IRS are carried out by incubating IRS-expressing cells in the presence and absence of a candidate compound and evaluating the level of IRS phosphorylation in the cells. A decrease in phosphorylation in cells cultured in the presence of the compound compared to in the absence of the compound indicates that the compound inhibits IRS-1 phosphorylation, and as a result, growth of HAAH-overexpressing tumors. Alternatively, such compounds are identified in an in vitro phosphorylation assay known in the art, e.g., one which measured phosphorylation of a synthetic substrate such as poly (Glu/Tyr).

#### EXAMPLE 1

##### Increased Expression of HAAH is Associated with Malignant Transformation

[0083] HAAH is a highly conserved enzyme that hydroxylates EGF-like domains in transformation associated proteins. The HAAH gene is overexpressed in many cancer types including human hepatocellular carcinomas and cholangiocarcinomas. HAAH gene expression was found to be undetectable during bile duct proliferation in both human disease and rat models compared to cholangiocarcinoma. Overexpression of HAAH in NIH-3T3 cells was associated with generation of a malignant phenotype, and enzymatic activity was found to be required for cellular transformation.

The data described below indicate that overexpression of HAAH is linked to cellular transformation of biliary epithelial cells.

[0084] To identify molecules that are specifically overexpressed in transformed malignant cells of human hepatocyte origin, the FOCUS hepatocellular carcinoma (HCC) cell line was used as an immunogen to generate monoclonal antibodies (mAb) that specifically or preferentially recognize proteins associated with the malignant phenotype. A lambda GT11 cDNA expression library derived from HepG2 HCC cells was screened, and a HAAH-specific mAb produced against the FOCUS cell line was found to recognize an epitope on a protein encoded by an HAAH cDNA. The HAAH enzyme was found to be upregulated in several different human transformed cell lines and tumor tissues compared to adjacent human tissue counterparts. The overexpressed HAAH enzyme in different human malignant tissues was found to be catalytically active.

[0085] HAAH gene expression was examined in proliferating bile ducts and in NIH 3T3 cells. Its role in the generation of the malignant phenotype was measured by the formation of transformed foci, growth in soft agar as an index of anchorage independent growth and tumor formation in nude mice. The role of enzymatic activity in the induction of transformed phenotype was measured by using a cDNA construct with a mutation in the catalytic site that abolished hydroxylase activity. The results indicated that an increase in expression of HAAH gene is associated with malignant transformation of bile ducts.

[0086] The following materials and methods were used to generate the data described below.

#### Antibodies

[0087] The FB50 monoclonal antibody was generated by cellular immunization of Balb/C mice with FOCUS HCC cells. A monoclonal anti-Dengue virus antibody was used as a non-relevant control. The HBOH2 monoclonal antibody was generated against a 52 kDa recombinant HAAH polypeptide and recognizes the catalytic domain of beta-hydroxylase from mouse and human proteins. Polyclonal anti-HAAH antibodies cross-react with rat hydroxylase protein. Control antibody anti-Erk-1 was purchased from Santa Cruz Biotechnology, Inc., Calif. Sheep anti-mouse and donkey anti-rabbit antisera labeled with horseradish peroxidase were obtained from Amersham, Arlington Heights, Ill.

#### Constructs

[0088] The murine full length AAH construct (pNH376) and the site-directed mutation construct (pNH376-H660) with abolished catalytic activity were cloned into the eukaryotic expression vector pcDNA3 (Invitrogen Corp., San Diego, Calif.). The full length human AAH was cloned into prokaryotic expression vector pBC-SK+ (Stratagene, La Jolla, Calif.). The full length human AAH (GENBANK Accession No. S83325) was subcloned into the EcoRI site of the pcDNA3 vector.

#### Animal Model of Bile Duct Proliferation

[0089] Rats were divided into 9 separate groups of 3 animals each except for group 9, which contained 5 rats. Group 1 was the non-surgical control group, and group 2

was the sham-operated surgical control. The remaining groups underwent common bile duct ligation to induce intrahepatic bile duct proliferation and were evaluated at 6, 12, 24, 48 hours and 4, 8 and 16 days as shown in Table 3. Animals were asphyxiated with CO<sub>2</sub>, and liver samples were taken from left lateral and median lobes, fixed in 2% paraformaldehyde and embedded in paraffin. Liver samples (5  $\mu$ m) were cut and stained with hematoxylin and eosin to evaluate intrahepatic bile duct proliferation. Immunohistochemistry was performed with polyclonal anti-HAAH antibodies that cross-react with the rat protein to determine levels of protein expression.

#### Bile Duct Proliferation Associated with Primary Sclerosing Cholangitis (PSC)

[0090] Liver biopsy samples were obtained from 7 individuals with PSC and associated bile duct proliferation. These individuals were evaluated according to standard gastroenterohepatological protocols. Patients were 22-46 years of age and consisted of 4 males and 3 females. Four had associated inflammatory bowel disease (3 ulcerative colitis and 1 Crohn's colitis). All patients underwent a radiological evaluation including abdominal ultrasonography and endoscopic retrograde cholangiopancreatography to exclude the diagnosis of extrahepatic biliary obstruction. Tissue sections were prepared from paraffin embedded blocks and were evaluated by hematoxylin and eosin staining for bile duct proliferation. Expression of HAAH was determined by immunohistochemistry using an HAAH-specific monoclonal antibody such as FB50.

#### Immunohistochemistry

[0091] Liver tissue sections (5  $\mu$ m) were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was quenched by a 30-minute treatment with 0.6% H<sub>2</sub>O<sub>2</sub> in 60% methanol. Endogenous biotin was masked by incubation with avidin-biotin blocking solutions (Vector Laboratories, Burlingame, Calif.). The FB50 mAb (for PSC samples) and polyclonal anti-HAAH-hydroxylase antibodies (for rat liver samples) were added to slides in a humidified chamber at 4° C. overnight. Immunohistochemical staining was performed using a standard avidin-biotin horseradish peroxidase complex (ABC) method using Vectastain Kits with diaminobenzidine (DAB) as the chromogen according to manufacturer's instructions (Vector Laboratories, Inc., Burlingame, Calif.). Tissue sections were counterstained with hematoxylin, followed by dehydration in ethanol. Sections were examined by a light microscopy for bile duct proliferation and HAAH protein expression. Paraffin sections of cholangiocarcinoma and placenta were used as positive controls, and hepatosteatosis samples were used as a negative controls. To control for antibody binding specificity, adjacent sections were immunostained in the absence of a primary antibody, or using non-relevant antibody to Dengue virus. As a positive control for tissue immunoreactivity, adjacent sections of all specimens were immunostained with monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase.

#### Western Blot Analysis

[0092] Cell lysates were prepared in a standard radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. The total amount of protein in the lysates was

determined by Bio-Rad colorimetric assay (Bio Rad, Hercules, Calif.) followed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, and subjected to Western blot analysis using FB50, HBOH2, anti-Erk-1 (used as an internal control for protein loading) as primary, sheep anti-mouse and donkey anti-rabbit antisera labeled with horseradish peroxidase as secondary antibodies. Antibody binding was detected with enhanced chemiluminescence reagents (SuperSignal, Pierce Chemical Company, Rockford, Ill.) and film autoradiography. The levels of immunoreactivity were measured by volume densitometry using NIH Image software.

#### Enzymatic Activity Assay

[0093] AAH activity was measured in cell lysates using the first EGF-like domain of bovine protein S as substrate where <sup>14</sup>C-labeled alpha-ketoglutarate hydroxylates the domain releasing <sup>14</sup>C containing CO<sub>2</sub> according to standard methods, e.g., those described by Jia et al., 1992, J. Biol. Chem. 267:14322-14327; Wang et al., 1991, J. Biol. Chem. 266:14004-14010; or Gronke et al., 1990, J. Biol. Chem. 265:8558-8565. Incubations were carried out at 37° C. for 30 min in a final volume of 40  $\mu$ l containing 48  $\mu$ g of crude cell extract protein and 75  $\mu$ M EGF substrate.

#### Cell Transfection Studies

[0094] The NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Washington, D.C.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma Chemical Co., St. Louis, Mo.), 1% L-glutamine, 1% non-essential amino acids and 1% penicillin-streptomycin (GIBCO BRL, Life Technologies, Inc., Grand Island, N.Y.). Subconfluent NIH-3T3 cells (3 $\times$ 10<sup>5</sup> cells/60-mm dish) were transfected with 10  $\mu$ g of one of the following plasmids: 1) non-recombinant pcDNA3 vector (Invitrogen Corp., San Diego, Calif.) as a negative control; 2) pNH376-H660, the murine AAH cDNA that was mutated in the catalytic domain and cloned into the pcDNA3 vector driven by a CMV promoter; 3) pNH376, the wild type murine AAH cDNA cloned into the pcDNA3 vector; 4) pCDHH, wild type human AAH cDNA cloned into the pcDNA3 vector; or 5) pLNCX-UP 1, a cDNA that encodes v-Src oncogene (positive control). Cells were transfected using the calcium phosphate transfection kit according to manufacturer's instructions (5 Prime-3 Prime, Inc., Boulder, Colo.). Comparison of cellular transfection efficiency was assessed with the various constructs. For this procedure, confluent plates obtained 48 hours after transfection were split and reseeded into 12 separate 6-cm dishes, and 6 of them were made to grow in the presence of 400  $\mu$ g/ml G-418 (GIBCO BRL, Life Technologies, Inc., Grant Island, N.Y.) containing medium. The number of G-418 resistant foci was determined at 14 days after transfection and used to correct for any variability in transfection efficiency.

#### Transformation Assay

[0095] The NIH-3T3 cells were transfected with the various constructs and allowed to reach confluence after 48 hours as described above. Each 6 cm dish was split and seeded into 12 different 6 cm dishes. While 6 of them were made to grow in the presence of G-418 to detect transfection efficiency, the other six were grown in complete medium

without G-418 and with a medium change every 4th day. The number of transformed foci were counted in these plates without G-418 and expressed as transformed foci per  $\mu\text{g}$  transfected DNA.

#### Anchorage-independent Cell Growth Assay

[0096] A limiting dilution technique (0.15 cell/well of a flat bottom 96-well-plate) was performed on transfectants grown in G-418 in order to isolate cell clones with different levels of HAAH activity as measured by Western blot analysis and enzymatic assay of hydroxylase activity. Cloned cell lines ( $1.0 \times 10^4$  cells) were suspended in complete medium containing 0.4% low-melting agarose (Sea-Plaque GTG Agarose; FMC Bioproducts, Rockland, Me.) and laid over a bottom agar mixture consisting of complete medium with 0.53% low-melting agarose. Each clone was assayed in triplicate. The clones were seeded under these conditions and 10 days later the size (positive growth  $>0.1$  mm in diameter) and number of foci were determined.

#### Tumorigenicity in Nude Mice

[0097] The same clones as assessed in the anchorage independent growth assay were injected into nude mice and observed for tumor formation. Tumorigenicity was evaluated using 10 animals in each of 4 groups (Charles River Labs., Wilmington, Mass.). Group 1 received  $1 \times 10^7$  cells stably transfected with mock DNA, Group 2-4 received  $1 \times 10^7$  cells of clones stable transfected with pNH376 and expressing various levels of murine HAAH protein. Nude mice were kept under pathogen-free conditions in a standard animal facility. Thirty days after tumor cell inoculation, the animals were sacrificed using isofluorane (Aerrane, Anaquest, N.J.) containing chambers and the tumors were carefully removed and weight determined.

#### Animal Model of Bile Duct Proliferation

[0098] Following ligation of the common bile duct, intrahepatic bile duct proliferation was evident at 48 hours. Tissue samples obtained 8 and 16 days following common bile duct ligation revealed extensive bile duct proliferation as shown in Table 3.

TABLE 3

| Bile duct proliferation and HAAH expression<br>at different intervals after common bile duct ligation |                        |                             |                      |
|-------------------------------------------------------------------------------------------------------|------------------------|-----------------------------|----------------------|
| Group                                                                                                 | Surgical Procedure     | Microscopy*                 | Immunohistochemistry |
| 1                                                                                                     | no surgery             | normal                      | negative             |
| 2                                                                                                     | sham surgery           | normal                      | negative             |
| 3                                                                                                     | 6 hours post ligation  | normal                      | negative             |
| 4                                                                                                     | 12 hours post ligation | normal                      | negative             |
| 5                                                                                                     | 24 hours post ligation | normal                      | negative             |
| 6                                                                                                     | 48 hours post ligation | minimal bile duct prolif.   | negative             |
| 7                                                                                                     | 4 days post ligation   | moderate bile duct prolif.  | negative             |
| 8                                                                                                     | 8 days post ligation   | extensive bile duct prolif. | negative             |
| 9                                                                                                     | 16 days post ligation  | extensive bile duct prolif. | negative             |

[0099] Investigation was performed under light microscopy following a hematoxylin and eosin staining.

[0100] Immunohistochemical staining failed to detect presence of HAAH in proliferating bile ducts at any time. Analysis of HAAH expression in bile ducts derived from sham surgical controls was also negative, while all samples exhibited positive immunoreactivity with control antibodies to glyceraldehyde 3-phosphate dehydrogenase. Thus, bile duct proliferation was not associated with increased HAAH expression in this standard animal model system.

#### HAAH Expression in PSC

[0101] The liver biopsy specimens from patients with PSC exhibited bile duct proliferation accompanied by periductal fibrosis and a mononuclear inflammatory cell infiltrate without evidence of dysplasia. Adjacent sections immunostained with the an HAAH-specific monoclonal antibody had no detectable HAAH immunoreactivity in proliferating bile ducts. In contrast, sections of cholangiocarcinoma that were immunostained simultaneously using the same antibody and detection reagents manifested intense levels of HAAH immunoreactivity in nearly all tumor cells, whereas adjacent sections of the cholangiocarcinomas exhibited a negative immunostaining reaction with monoclonal antibody to Dengue virus. These findings indicate that HAAH expression was associated with malignant transformation rather than non-cancerous cellular proliferation of intrahepatic bile ducts.

#### HAAH Associated Transformation of NIH-3T3 Cells

[0102] The transforming capability of the murine and human AAH genes, as well as the murine AAH mutant construct without enzymatic activity were compared to mock DNA (negative control) and v-Src transfected NIH-3T3 cells (positive control). The transforming capability of murine AAH was found to be 2-3 times that of vector DNA control as shown in FIG. 1. The transforming capacity of the human gene was greater than that observed with the murine AAH ( $32 \pm 1.5$  versus  $13 \pm 2.6$  transformed foci, respectively). The murine and human AAH transfected cells formed large foci, resembling those of v-Src transfected fibroblasts, compared to the occasional much smaller foci observed in cells transfected with vector DNA that displayed the contact inhibition of fibroblast cell lines. Parallel experiments performed using the mutant pNH376-H660 construct without enzymatic activity revealed no transforming activity. This finding indicates that the enzymatic activity of HAAH is required for the transforming activity exhibited by the HAAH gene.

#### Anchorage-independent Cell Growth Assay

[0103] After transient transfection with the murine AAH construct, several different transformed foci were isolated for dilutional cloning experiments to establish stable transfected cell clones with different levels of HAAH gene expression. Nine different cloned cell lines were selected for further study. The expression level of the HAAH protein was determined by Western blot analysis. Clones 7 and 18 had a modest increase in HAAH protein expression, yet formed large colonies in soft agar (FIG. 2). Protein loading was equivalent in all lanes as shown by immunoblotting of the

same membranes with an anti-Erk-1 monoclonal antibody. The increased protein expression was associated with increased enzymatic activity as shown in **FIG. 3**. The capability of these clones to exhibit anchorage independent cell growth in soft agar is presented in **FIG. 3**. All 3 clones with increased HAAH gene expression demonstrated anchorage independent cell growth compared to the mock DNA transfected clone.

#### Tumor Formation in Nude Mice

**[0104]** The 3 clones with increased HAAH gene expression were evaluated for the ability to form tumors in nude mice. Tumor size in the mouse given clone 18 was compared to a mock DNA transfected clone. Clones 7, 16 and 18 were highly transformed in this assay and produced large tumors with a mean weight of 2.5, 0.9 and 1.5 grams, respectively (**FIG. 4**). These data indicate that overexpression of HAAH contributes to induction and maintenance of the malignant phenotype *in vivo*.

#### High Level HAAH Expression is Indicative of Malignancy

**[0105]** In order to determine if HAAH expression was associated with malignancy rather than increased cell turnover, two models of bile duct proliferation were studied. In the animal model, ligation of the common bile duct induced extensive intrahepatic bile duct proliferation, yet there was no evidence of HAAH gene expression under these experimental conditions as shown in Table 3. Similarly, HAAH gene expression was assessed in a human disease model associated with bile duct proliferation since PSC is an autoimmune liver disease associated with destruction as well as proliferation of the intra and extrahepatic bile ducts. PSC is premalignant disease, and a significant proportion of affected individuals will eventually develop cholangiocarcinoma. However, no evidence for increased HAAH gene expression in the presence of extensive bile duct proliferation.

**[0106]** Having established that HAAH protein levels were elevated in cholangiocarcinoma and not in normal or proliferating bile ducts, the role of HAAH in the generation of a malignant phenotype was studied. The HAAH gene was transfected into NIH-3T3 cells and cellular changes, e.g., increased formation of transformed foci, colony growth in soft agar and tumor formation in nude mice associated with malignant transformation, were evaluated. The full-length murine and human AAH genes were cloned into expression constructs and transiently transfected into NIH-3T3 cells. An increased number of transformed foci was detected in cells transfected both with the murine and human AAH genes as compared to mock DNA transfected controls. The increased number of transformed foci, after controlling for transfection efficiency, was not as high compared to v-Src gene transfected cells used as a positive control. The enzymatic activity of the HAAH gene was required for a malignant phenotype because a mutant construct which abolished the catalytic site had no transforming properties. Several stable transfectants and cloned NIH-3T3 cell lines with a modest increase in HAAH protein levels and enzymatic activity were established. Such cell lines were placed in soft agar to examine anchorage independent cell growth as another property of the malignant phenotype. All cell lines grew in soft agar compared to mock DNA transfected

control, and there was a positive correlation between the cellular level of HAAH gene expression and the number and size of colonies formed. Three of these cloned cell lines formed tumors in nude mice. All three cell lines with increased HAAH expression were oncogenic as shown by the development of large tumors as another well-known characteristic of the transformed phenotype.

**[0107]** To determine whether cellular changes induced by overexpression of HAAH were related to the enzymatic function, a site-directed mutation was introduced into the gene that changed the ferrous iron binding site from histidine to lysine at 660th position of mouse HAAH thereby abolishing hydroxylase activity of the murine HAAH. A corresponding mutation in HAAH is used as a dominant negative mutant to inhibit HAAH hydroxylase activity. The pNH376-H660 construct had no transformation activity indicating cellular changes of the malignant phenotype induced by overexpression depends on the enzymatic activity of the protein.

**[0108]** Notch receptors and their ligands have several EGF-like domains in the N-terminal region that contain the putative consensus sequence for beta-hydroxylation. Notch ligands are important elements of the Notch signal transduction pathway and interaction of Notch with its ligands occurs by means of EGF-like domains of both molecules. Point mutations affecting aspartic acid or asparagine residues in EGF-like domains that are the targets for beta-hydroxylation by HAAH reduce calcium binding and protein-protein interactions involved in the activation of downstream signal transduction pathways. Overexpression of HAAH and Notch protein hydroxylation by HAAH contributes to malignancy. Tumor growth is inhibited by decreasing Notch protein hydroxylation by HAAH.

**[0109]** The data presented herein is evidence that high-level HAAH expression is linked to malignant transformation. An increase in expression of the HAAH cDNA in NIH-3T3 cells induced a transformed phenotype manifested by increased numbers of transformed foci, anchorage-independent growth, and tumorigenesis in nude mice. In addition, intact HAAH-enzyme was found to be required for HAAH-associated transformation. Accordingly, inhibition of as little as 20% of endogenous HAAH enzymatic activity or expression confers a therapeutic benefit. For example, clinical benefit is achieved by 50%-70% inhibition of HAAH expression or activity after administration of an HAAH inhibitory compound compared to the level associated with untreated cancer cell or a normal noncancerous cell.

**[0110]** HAAH is regulated at the level of transcription. Only modest increases in HAAH expression and enzyme activity were required for cellular transformation. These results indicate that increased HAAH gene expression and enzyme activity contribute to the generation or maintenance of the transformed phenotype and that decreasing transcription of the HAAH gene or decreasing enzymatic activity of the HAAH gene product leads to a decrease in malignancy. Accordingly, HAAH transcription is inhibited by administering compounds which decrease binding of Fos and/or Jun (elements which regulate HAAH transcription) to HAAH promoter sequences.

**[0111]** Since HAAH is up-regulated with malignant transformation of bile duct epithelium, and HAAH immunoreactivity is detectable on tumor cell surface membranes,

HAAH is also a molecule to which to target a cytotoxic agent, e.g., by linking the cytotoxic agent to a compound that binds to HAAH expressed on the surface of a tumor cell. Assay of HAAH protein levels in either biological fluids such as bile, or cells obtained by fine needle aspiration is a diagnostic marker of human cholangiocarcinoma.

#### EXAMPLE 2

##### Expression of AAH and Growth and Invasiveness of Malignant CNS Neoplasms

[0112] AAH is abundantly expressed in carcinomas and trophoblastic cells, but not in most normal cells, including those of CNS origin. High levels of AAH expression were observed in 15 of 16 glioblastomas, 8 of 9 anaplastic oligodendrogliomas, and 12 of 12 primitive neuroectodermal tumors (PNETs). High levels of AAH immunoreactivity were primarily localized at the infiltrating edges rather than in the central portions of tumors. Double-label immunohistochemical staining demonstrated a reciprocal relationship between AAH and tenascin, a substrate for AAH enzyme activity. PNET2 neuronal cell lines treated with phorbol ester myristate or retinoic acid to stimulate neuritic extension and invasive growth exhibited high levels of AAH expression, whereas  $H_2O_2$ -induced neurite retraction resulted in down-regulation of AAH. PNET2 neuronal cells that stably over-expressed the human AAH cDNA had increased levels of PCNA and Bcl-2, and reduced levels of p21/Waf1 and p16, suggesting that AAH overexpression results in enhanced pathological cell proliferation, cell cycle progression, and resistance to apoptosis. In addition, the reduced levels of p16 observed in AAH-transfectants indicate that AAH over-expression confers enhanced invasive growth of neoplastic cells since deletion or down-regulation of the p16 gene correlates with more aggressive and invasive in vivo growth of glioblastomas. Increased AAH immunoreactivity was detected at the infiltrating margins of primary malignant CNS neoplasms, further indicating a role of HAAH in tumor invasiveness.

[0113] The following materials and methods were used to generate the data described below.

##### Analysis of AAH Immunoreactivity in Primary Human Malignant CNS Neoplasms

[0114] AAH immunoreactivity was examined in surgical resection specimens of glioblastoma (N=16), anaplastic oligodendroglioma (N=9), and primitive neuroectodermal tumor (PNET; supratentorial neuroblastomas (N=3) and medulloblastomas (N=9). The histopathological sections were reviewed to confirm the diagnoses using standard criteria. Paraffin sections from blocks that contained representative samples of viable solid tumor, or tumor with adjacent intact tissue were studied. Sections from normal adult postmortem brains (N=4) were included as negative controls. AAH immunoreactivity was detected using qn HAAH-specific monoclonal antibody. Immunoreactivity was revealed by the avidin-biotin horseradish peroxidase complex method (Vector ABC Elite Kit; Vector Laboratories, Burlingame, Calif.) using 3-3' diaminobenzidine (DAB) as the chromogen (24) and hematoxylin as a counterstain.

[0115] Tenascin and laminin are likely substrates for AAH due to the presence of EGF-like repeats within the mol-

ecules. Double-immunostaining studies were performed to co-localize AAH with tenascin or laminin. The AAH immunoreactivity was detected by the ABC method with DAB as the chromogen, and tenascin or laminin immunoreactivity was detected by the avidin-biotin alkaline phosphatase complex method (Vector Laboratories, Burlingame, Calif.) with BCIP/NBT as the substrate. As positive and negative controls, adjacent sections were immunostained with monoclonal antibody to glial fibrillary acidic protein (GFAP) and Hepatitis B surface antigen. All specimens were batch immunostained using the same antibody dilutions and immunodetection reagents.

##### Cell Lines and Culture Conditions

[0116] Studies were conducted to determine whether AAH expression was modulated with neurite (filopodia) extension (sprouting) as occurs with invasive growth of malignant neoplasms. Human PNET2 CNS-derived and SH-Sy5y neuroblastoma cells were cultured and stimulated for 0, 1, 2, 3, 5, or 7 days with 100 nM phorbol 12-ester 13-acetate or 10  $\mu$ M retinoic acid to induce sprouting. In addition, to examine the effects of neurite retraction on AAH expression, subconfluent cultures were treated for 24 hours with low concentrations (10-40  $\mu$ M) of  $H_2O_2$ . For both studies, AAH expression was evaluated by Western blot analysis using the an HAAH-specific antibody.

##### Generation of PNET2 AAH-transfected Clones

[0117] The full-length human AAH cDNA (SEQ ID NO:3) was ligated into the pcDNA3.1 mammalian expression vector in which gene expression was under the control of a CMV promoter (Invitrogen Corp., San Diego, Calif.). PNET2 cells were transfected with either pHAAH or pcDNA3 (negative control) using Cellfectin reagent (Gibco BRL, Grand Island, N.Y.). Neomycin-resistant clones were selected for study if the constitutive levels of AAH protein expression were increased by at least two-fold relative to control (pcDNA3) as detected by Western blot analysis. To determine how AAH overexpression altered the expression of genes that modulate the transformed phenotype, the levels of proliferating cell nuclear antigen (PCNA), p53, p21/Waf1, Bcl-2, and p16 were measured in cell lysates prepared from subconfluent cultures of AAH (N=5) and pcDNA3 (N=5) stably transfected clones. PCNA was used as marker of cell proliferation. p53, p21/Waf1, and Bcl-2 levels were examined to determine whether cells that over-expressed AAH were more prone to cell cycle progression and more resistant to apoptosis. The levels of p16 were assessed to determine whether AAH over-expression has a role in tumor invasiveness.

##### Western Blot Analysis

[0118] Cells grown in 10 cm<sup>2</sup> dishes were lysed and homogenized in a standard radioimmunoprecipitation assay RIPA buffer containing protease and phosphatase inhibitors. The supernatants collected after centrifuging the samples at 12,000×g for 10 minutes to remove insoluble debris were used for Western blot analysis. Protein concentration was measured using the BCA assay (Pierce Chemical Co, Rockford, Ill.). Samples containing 60  $\mu$ g of protein were electrophoresed in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and subjected to Western blot analysis. Replicate blots were probed with the individual antibodies.

Immunoreactivity was detected with horseradish peroxidase conjugated IgG (Pierce Chemical Co, Rockford, Ill.) and enhanced chemiluminescence reagents. To quantify the levels of protein expression, non-saturated autoradiographs were subjected to volume densitometry using NIH Image software, version 1.6. Statistical comparisons between pHAH and pcDNA3 transfected cells were made using Student T tests.

#### Antibodies

[0119] HAAH-specific monoclonal antibody generated against the FOCUS hepatocellular carcinoma cells were used to detect AAH immunoreactivity. Monoclonal antibodies to tenascin, and glial fibrillary acidic protein, and rabbit polyclonal antibody to laminin were purchased from Sigma Co. (St. Louis, Mo.). Rabbit polyclonal antibody to human p16 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.). The 5C3 negative control monoclonal antibody to Hepatitis B surface antigen was generated using recombinant protein and used as a negative control.

#### AAH Immunoreactivity in Primary Malignant Trains Tumors

[0120] AAH immunoreactivity was detected in 15 of 16 glioblastomas, 8 of 9 anaplastic oligodendrogliomas, and all 12 PNETs. AAH immunoreactivity was localized in the cytoplasm, nucleus, and cell processes. The tissue distribution of AAH immunoreactivity was notable for the intense labeling localized at the interfaces between tumor and intact brain, and the conspicuously lower levels of immunoreactivity within the central portions of the tumors. High levels of AAH immunoreactivity were also observed in neoplastic cells distributed in the subpial zones, leptomeninges, Virchow-Robin perivascular spaces, and in individual or small clusters of neoplastic cells that infiltrated the parenchyma. In contrast, AAH immunoreactivity was not detectable in normal brain. The distribution of AAH immunoreactivity appeared not to be strictly correlated with DNA synthesis since the density of nuclei in mitosis (1-5%) was similar in the central and peripheral portions of the tumors.

#### Relationship Between AAH and Tenascin Immunoreactivity in Glioblastomas

[0121] Tenascin is an extracellular matrix-associated antigen expressed in malignant gliomas. Tenascin contains EGF-like domains within the molecule, a substrate for HAAH hydroxylation. To localize AAH in relation to tenascin immunoreactivity in malignant brain tumors, double-label immunohistochemical staining was performed in which AAH was detected using a brown chromogen (DAB), and tenascin, a blue chromogen (BCIP/NBT). Adjacent sections were similarly double-labeled to co-localize AAH with laminin, another EGF domain containing extracellular matrix molecule expressed in the CNS. Intense levels of tenascin immunoreactivity were observed in perivascular connective tissue and in association with glomeruloid proliferation of endothelial cells. The double-labeling studies demonstrated a reciprocal relationship between AAH and tenascin immunoreactivity such that high levels of AAH were associated with low or undetectable tenascin, and low levels of AAH were associated with abundant tenascin immunoreactivity. Although laminins are also likely substrates for AAH enzyme activity due to the EGF repeats

within the molecules, double labeling studies revealed only low levels of laminin immunoreactivity throughout the tumors and at interfaces between tumor and intact tissue.

#### Analysis of AAH Expression in Neuronal Cell Lines Treated with PMA or RA

[0122] Neuritic sprouting/filopodia extension marks invasive growth of neoplastic neuronal cells. PMA activates protein kinase C signal transduction pathways that are involved in neuritic sprouting. Retinoic acid binds to its own receptor and the ligand-receptor complex translocates to the nucleus where it binds to specific consensus sequences present in the promoter/enhancer regions of target genes involved in neuritic growth. Both PNET2 and SH-Sy5y cells can be induced to sprout by treatment with PMA (60-120 nM) or retinoic acid (5-10  $\mu$ M). FIGS. 5A-D depict data from representative Western blot autoradiographs; the bar graphs correspond to the mean  $\pm$  S.D. of results obtained from three experiments. Western blot analysis with the FB50 antibody detected doublet bands corresponding to protein with an molecular mass of approximately 85 kDa. Untreated PNET2 cells had relatively low levels of AAH immunoreactivity (FIG. 5A), whereas untreated SH-Sy5y cells had readily detected AAH expression (FIG. 5B). Untreated PNET2 cells exhibited polygonal morphology with coarse, short radial cell processes, whereas SH-Sy5y cells were slightly elongated and spontaneously extend fine tapered processes. Both cell lines manifested time-dependent increases in the levels of AAH immunoreactivity following either RA (FIGS. 5A and 5B) or PMA (FIG. 5C) stimulation and neurite extension. In PNET2 cells, the levels of AAH protein increased by at least two-fold 24 hours after exposure to RA or PMA, and high levels of AAH were sustained throughout the 7 days of study. In SH-Sy5y cells, the RA- or PMA-stimulated increases in AAH expression occurred more gradually and were highest after 7 days of treatment (FIG. 5B).

[0123] To examine the effect of AAH expression on neurite retraction, PNET2 and SH-Sy5y cells were treated with low concentrations (8-40  $\mu$ M) of  $H_2O_2$ . After 24 hours exposure to up to 40  $\mu$ M  $H_2O_2$ , although most cells remained viable (Trypan blue dye exclusion), they exhibited neurite retraction and rounding. Western blot analysis using the FB50 antibody demonstrated  $H_2O_2$  dose-dependent reductions in the levels of AAH protein (FIG. 5D).

#### Effects of AAH Over-expression in PNET2 Cells

[0124] To directly assess the role of AAH overexpression in relation to the malignant phenotype, PNET2 cells were stably transfected with the human full-length cDNA with gene expression under control of a CMV promoter (pHAH). Neomycin-resistant clones that had at least two-fold higher levels of AAH immunoreactivity relative to neomycin-resistant pcDNA3 (mock) clones were studied. Since aggressive behavior of malignant neoplasms is associated with increased DNA synthesis, cell cycle progression, resistance to apoptosis, and invasive growth, the changes in phenotype associated with constitutive over-expression of AAH were characterized in relation to PCNA, p21/Waf1, p53, Bcl-2, and p16. PCNA was used as an index of DNA synthesis and cell proliferation. p21/Waf1 is a cell cycle inhibitor. Expression of the p53 tumor-suppressor gene increases prior to apoptosis, whereas bcl-2 inhibits apoptosis

and enhances survival of neuronal cells. p16 is an oncosuppressor gene that is often either down-regulated or mutated in infiltrating malignant neoplasms.

**[0125]** Five pHAHA and 5 pcDNA3 clones were studied. Increased levels of AHA expression in the pHAHA transfected clones was confirmed by Western (**FIG. 6**) and Northern blot analyses. Western blot analysis using cell lysates from cultures that were 70 to 80 percent confluent demonstrated that constitutively increased levels of AHA expression (approximately 85 kDa;  $P < 0.05$ ) in pHAHA-transfected cells were associated with significantly increased levels of PCNA (approximately 35 kDa;  $P < 0.01$ ) and Bcl-2 (approximately 25 kDa;  $P < 0.05$ ), and reduced levels of p21/Waf1 (approximately 21 kDa;  $P < 0.001$ ) and p16 (approximately 16 kDa;  $P < 0.001$ ) (**FIG. 6**). However, the pHAHA stable transfectants also exhibited higher levels of wild-type p53 (approximately 53-55 kDa). Although AHA expression (85 kDa protein) in the stable transfectants was increased by only 75 to 100 percent, the levels of p16 and p21/Waf1 were sharply reduced, and PCNA increased by nearly two-fold (**FIG. 6**).

#### Increased AHA Expression is Indicative of Growth and Invasiveness of Malignant CNS Neoplasms

**[0126]** The data described herein demonstrates that AHA overexpression is a diagnostic tool by which to identify primary malignant CNS neoplasms of both neuronal and glial cell origin. Immunohistochemical staining studies demonstrated that AHA overexpression was detectable mainly at the interfaces between solid tumor and normal tissue, and in infiltrating neoplastic cells distributed in the subpial zones, leptomeninges, perivascular spaces, and parenchyma. In vitro experiments demonstrated that AHA gene expression was modulated with neurite (filopodium) extension and invasiveness and down-regulated with neurite retraction. In addition, PNET2 cells stably transfected with the AHA cDNA exhibited increased PCNA and bcl-2, and reduced Waf1/p21 and p16 expression. Therefore, AHA overexpression contributes to the transformed phenotype of CNS cells by modulating the expression of other genes that promote cellular proliferation and cell cycle progression, inhibit apoptosis, or enhance tumor cell invasiveness.

**[0127]** The data demonstrated readily detectable AHA mRNA transcripts (4.3 kDa and 2.6 kDa) and proteins (85 kDa and 50-56 kDa) in PNET2 and SH-Sy5y cells, but not in normal brain. Correspondingly, high levels of AHA immunoreactivity were observed in 35 of the 37 in malignant primary CNS-derived neoplasms studied, whereas the 4 normal control brains had no detectable AHA immunoreactivity. The presence of high-level AHA immunoreactivity at the infiltrating margins and generally not in the central portions of the tumors indicates that AHA overexpression is involved in the invasive growth of CNS neoplasms. Administration of compounds which decrease AHA expression or enzymatic activity inhibits proliferation of CNS tumors which overexpress AHA, as well as metastases of CNS tumors to other tissue types.

**[0128]** The AHA enzyme hydroxylates EGF domains of a number of proteins. Tenascin, an extracellular matrix molecule that is abundantly expressed in malignant gliomas, contains EGF-like domains. Since tenascin promotes tumor cell invasion, its abundant expression in glioblastomas rep-

resents an autocrine mechanism of enhanced tumor cell growth vis-a-vis the frequent overexpression of EGF or EGF-like receptors in malignant glial cell neoplasms. Analysis of the functional domains of tenascins indicated that the mitogenic effects of this family of molecules are largely mediated by the fibronectin domains, and that the EGF-like domains inhibit growth, cell process elongation, and matrix invasion. Therefore, hydroxylation of the EGF-like domains by AHA represents an important regulatory factor in tumor cell invasiveness.

**[0129]** Double-label immunohistochemical staining studies demonstrated a reciprocal relationship between AHA and tenascin immunoreactivity such that high levels AHA immunoreactivity present at the margins of tumors were associated with low levels of tenascin, and low levels of AHA were often associated with high levels of tenascin. These observations indicated that AHA hydroxylation of EGF-like domains of tenascin alters the immunoreactivity of tenascin protein, and in so doing, facilitates the invasive growth of malignant CNS neoplasms into adjacent normal tissue and perivascular spaces.

**[0130]** AHA immunoreactivity was examined in PNET2 and SH-Sy5y neuronal cells induced to undergo neurite extension with PMA or retinoic acid, or neurite retraction by exposure to low doses of  $H_2O_2$ . AHA expression was sharply increased by PMA- or retinoic acid-induced neurite (filopodium) extension, and inhibited by  $H_2O_2$ -induced neurite retraction and cell rounding. Neurite or filopodium extension and attachment to extracellular matrix are required for tumor cell invasion in the CNS. The EGF-like domains of tenascin inhibit neuritic and glial cell growth into the matrix during development.

**[0131]** To directly examine the role of AHA overexpression in relation to the transformed phenotype, genes modulated with DNA synthesis, cell cycle progression, apoptosis, and tumor invasiveness were examined in neuronal cell clones that stably over-expressed the human AHA cDNA. The findings of increased PCNA and reduced Waf1/p21 immunoreactivity indicated that AHA overexpression enhances cellular proliferation and cell cycle progression. In addition, the finding of increased Bcl-2 expression indicated that AHA overexpression contributes to the transformed phenotype by increasing cellular resistance to apoptosis. The apparently contradictory finding of higher levels of p53 in the cells that overexpressed AHA is explained by the observation that high levels of wildtype p53 in immature neuronal cells were associated with neuritic growth (invasiveness) rather than apoptosis. Levels of p16 were reduced (compared to normal cells) or virtually undetectable in cells that constitutively overexpressed AHA; a deletion mutation of the p16 gene has been correlated with invasive growth and more rapid progression of malignant neoplasms, including those of CNS origin. These data indicate that p16 expression is modulated by AHA.

#### EXAMPLE 3

##### Increased AHA Production and IRS-mediated Signal Transduction

**[0132]** IRS-1 mediated signal transduction pathway is activated in 95% of human HCC tumors compared to the adjacent uninvolved liver tissue. AHA is a downstream

effector gene involved in this signal transduction pathway. HAAH gene upregulation is closely associated with overexpression of IRS-1 in HCC tumors as revealed by immunohistochemical staining and Western blot analysis. A high level of HAAH protein is expressed in HCC and cholangiocarcinoma compared to normal hepatocytes and bile ducts. Both of these tumors also exhibit high level expression of IRS-1 by immunohistochemical staining. FOCUS HCC cell clones stably transfected with a C-terminal truncated dominant negative mutant of IRS-1, which blocks insulin and IGF-1 stimulated signal transduction, was associated with a striking reduction in HAAH gene expression in liver. In contrast, transgenic mice overexpressing IRS-1 demonstrate an increase in HAAH gene expression by Western blot analysis. Insulin stimulation of FOCUS HCC cells (20 and 40 U) in serum free medium and after 16 hr of serum starvation demonstrated upregulation of HAAH gene expression. These data indicate that HAAH gene expression is a downstream effector of the IRS-1 signal transduction pathway.

#### EXAMPLE 4

##### Effects of HAAH Expression Levels on the Characteristics of the Malignant Phenotype

[0133] Overexpression of IRS-1 in NIH 3T3 cells induces transformation. The full-length murine HAAH construct was cloned into the pcDNA3 eukaryotic expression vector. A second murine construct encoded HAAH with abolished catalytic activity due to a site directed mutation. The full-length human HAAH cDNA was cloned into the pcDNA3 expression vector as well as a plasmid that encodes v-src which was used as a positive control for transformation activity. Standard methods were used for transfection of NIH 3T3 cells, control for transfection efficiency, assays of HAAH enzymatic activity, transformation by analysis of foci formation, anchorage-independent cell growth assays and analysis of tumorigenicity in nude mice. The data indicated that HAAH overexpression is associated with generation of a malignant phenotype.

TABLE 4

| Overexpression of enzymatically active HAAH indicates malignancy |                                   |                       |                            |
|------------------------------------------------------------------|-----------------------------------|-----------------------|----------------------------|
| cDNA                                                             | # of foci $\pm$ S.D. <sup>b</sup> | NIH 3T3 clone         | # of colonies <sup>c</sup> |
| pcDNA3 (mock)                                                    | 6.0 $\pm$ 3.3                     | PcDNA (mock)          | 0.4 $\pm$ 0.5              |
| murine HAAH                                                      | 14.0 $\pm$ 2.9                    | clone 18 <sup>d</sup> | 6.2 $\pm$ 2.9              |
| mutant murine HAAH <sup>a</sup>                                  | 1.6 $\pm$ 1.0                     | clone 16 <sup>e</sup> | 4.7 $\pm$ 6.5              |
| human HAAH                                                       | 32.0 $\pm$ 5.4                    |                       |                            |
| v-src                                                            | 98.0 $\pm$ 7.1                    |                       |                            |

<sup>a</sup>enzymatically inactive HAAH

<sup>b</sup>P < 0.01 compared to mock and mutant murine HAAH

<sup>c</sup>P < 0.001 compared to mock

<sup>d</sup>Clone 18 is a stable cloned NIH 3T3 cell line that overexpresses human HAAH by approximately two fold.

<sup>e</sup>Clone 16 is a stable cloned NIH 3T3 cell line that overexpresses human HAAH by about 50%.

[0134] These data indicate that overexpression of HAAH is associated with formation of transformed foci. Enzymatic activity is required for cellular transformation to occur.

Cloned NIH 3T3 cell lines with increased human HAAH gene expression grew as solid tumors in nude mice. HAAH is a downstream effector gene of the IRS-1 signal transduction pathway.

#### EXAMPLE 5

##### Inhibition of HAAH Gene Expression

[0135] The FOCUS HCC cell line from which the human HAAH gene was initially cloned has a level of HAAH expression that is approximately 3-4 fold higher than that found in normal liver. To make an HAAH antisense construct, the full length human HAAH cDNA was inserted in the opposite orientation into a retroviral vector containing a G418 resistant gene, and antisense RNA was produced in the cells. Shorter HAAH antisense nucleic acids, e.g., those corresponding to exon 1 of the HAAH gene are also used to inhibit HAAH expression.

[0136] FOCUS cells were infected with this vector and the level of HAAH was determined by Western blot analysis. A reduction in HAAH gene expression was observed. Growth rate and morphologic appearance of cells infected with a retrovirus containing a nonrelevant Green Fluorescent Protein (GFP) also inserted in the opposite orientation as a control (FIG. 8). Cells (harboring the HAAH antisense construct) exhibited a substantial change in morphology characterized by an increase in the cytoplasm to nuclear ratio as well as assuming cell shape changes that were reminiscent of normal adult hepatocytes in culture. Cells with reduced HAAH levels grew at a substantially slower rate than retroviral infected cells expressing antisense (GFP) (control) as shown in FIG. 8. A reduction in HAAH gene expression was associated with a more differentiated non-cancerous "hepatocyte like" phenotype. Expression of HAAH antisense sequences are used to inhibit tumor growth rate. Reduction of HAAH cellular levels results in a phenotype characterized by reduced formation of transformed foci, low level or absent anchorage independent growth in soft agar, morphologic features of differentiated hepatocytes as determined by light and phase contrast microscopy, and no tumor formation (as tested by inoculating the cells into nude mice).

#### EXAMPLE 6

##### Inhibition of AAH Expression by AAH Antisense Oligonucleotides

[0137] Oligonucleotides that inhibit AAH gene expression were designed and synthesized using standard methods. For example, antisense oligonucleotides (20 mers) were designed to bind to the 5' region of the AAH mRNA and overlap with the AUG initiation codon (Table 5). The antisense oligonucleotides were selected such that they were complementary to sequences beginning 1 (Location -1), 6 (Location -6), or 11 (Location -11) nucleotides upstream (prior to) the "A" of the AUG (methionine) codon. In addition, a sense oligonucleotide beginning at Location -3 was made.

TABLE 5

| Sequence of exemplary oligonucleotide molecules |                 |
|-------------------------------------------------|-----------------|
| Location (-1)                                   |                 |
| 5' CAT TCT TAC GCT GGG CCA TT 3'                | (SEQ ID NO: 10) |
| Location (-6)                                   |                 |
| 5' TTA CGC TGG GCC ATT GCA CG 3'                | (SEQ ID NO: 11) |
| Location (-11)                                  |                 |
| 5' CTG GGC CAT TGC ACG GTC CG 3'                | (SEQ ID NO: 12) |
| Sense                                           |                 |
| 5' ATC ATG CAA TGG CCC AGC GTA A                | (SEQ ID NO: 13) |
| 3'                                              |                 |

**[0138]** AAH antisense oligonucleotides tested were found to inhibit AAH gene expression. Using an in vitro cell free transcription translation assay (TNT Quick Coupled System), the human AAH cDNA (pHAAH) was used to synthesize AAH protein. In vitro translation was achieved with rabbit reticulocyte lysate included in the reaction mixture. The translated product was labeled with [<sup>35</sup>S] methionine in the presence of reaction buffer, RNA polymerase, amino acid mixture, and ribonuclease inhibitor (RNasin). The products were analyzed by SDS-PAGE followed by autoradiography. A luciferase (Luc) expressing plasmid was used as a positive control. In the second and third lanes, synthesis of the ~85 kD AAH protein is shown (AAH, arrow) using 1 or 2 micrograms of plasmid as the template and the T7 DNA-dependent RNA polymerase primer/promoter to generate mRNA. The addition of 100× or 1000× excess antisense oligonucleotide primer resulted in progressively greater degrees of inhibition of AAH protein synthesis, whereas the inclusion of the same amounts of sense oligonucleotide had no effect on AAH protein synthesis. Further studies demonstrated complete inhibition of AAH protein synthesis only with the antisense oligonucleotides. In addition, effective inhibition of gene expression was observed using all three antisense oligonucleotides tested.

**[0139]** Inhibition of AAH gene expression was also tested in cells. Substantial reduction in HAAH gene expression was detected by simply adding the antisense oligonucleotides to the culture medium of the cells. The MILQ assay quantifies in situ hybridization binding in cultured cells without the need for RNA extraction. The MILQ assay was used to study competitive antisense binding inhibition to illustrate that the antisense probe hybridized to the mRNA expressed endogenously within the Sh-SySy neuroblastoma cells. In this figure, inhibition of FITC-labeled Location -6 antisense oligonucleotide binding using specific unlabeled antisense oligonucleotides is shown. Minimal inhibition of binding was observed using non-relevant oligonucleotides. The unlabeled specific oligonucleotide was capable of effectively competing for the binding site designated by the FITC-conjugated Location -6 probe, whereas the non-relevant probe exhibited significantly less inhibition at the same molar concentration. Bound probe (FITC-labeled) was detected using horseradish peroxidase conjugated antibodies to FITC, and luminescence reagents were used to detect the bound antibody. Luminescence units were corrected for cell density and are arbitrary in nature. These data indicate that cells effectively take up antisense oligonucleotides in the

surrounding environment and that the oligonucleotides taken up effectively and specifically inhibit HAAH gene expression.

**[0140]** Inhibition of HAAH gene expression is enhanced by contacting cells with a phosphorothioate derivative of the HAAH antisense. Phosphorothioate antisense derivatives are made using methods well known in the art. The MILQ assay was used to measure gene expression resulting from antisense oligonucleotide gene delivery. Cells were contacted with AAH Location -6 antisense DNA, and AAH protein expression was measured using methods known in the art, e.g., the MICE assay (de la Monte, et al, 1999, Biotechniques), to determine if it was inhibited by hybridization with the oligonucleotide. The MICE assay is used to measure immunoreactivity in cultured cells without the need to extract proteins or perform gel electrophoresis. This assay is more sensitive than Western blot analysis. Using the MICE assay, AAH immunoreactivity was assessed in cells transfected with non-relevant (random) oligonucleotide sequences, specific antisense oligonucleotides (Location -6), and a phosphorothioate Location -6 antisense oligonucleotide. Phosphorothioate chemical modification of the oligonucleotide was found to permit greater stability of the DNA inside the cell since the sulfur group protects the DNA from the degradation that normally occurs with phosphodiester bonds and cellular nucleases. Antisense AAH oligonucleotide (Location -6) transfection resulted in reduced levels of AAH immunoreactivity, and using the phosphorothioate linked Location -6 antisense oligonucleotide, the effect of inhibiting AAH gene expression was substantial relative to the levels observed in cells transfected with the random oligonucleotide. The more effective inhibition of AAH expression using the phosphorothioate-linked antisense oligonucleotide was likely due to the greater stability of the molecule combined, with retained effective binding to mRNA.

#### EXAMPLE 7

##### Human IRS-1 Mutants

**[0141]** Insulin/IGF-1 stimulated expression of HAAH in HCC cell lines. Dominant-negative IRS-1 cDNAs mutated in the plextrin and phosphotyrosine (PTB) domains, and Grb2, Syp and PI3K binding motifs located in the C-terminus of the molecule were constructed. Human IRS-1 mutant constructs were generated to evaluate how HAAH gene expression is upregulated by activation of the IRS-1 growth factor signal transduction cascade. Specific mutations in the C terminus of the hIRS-1 molecule abolished the various domains which bind to SH2-effector proteins such as Grb2, Syp and PI3K. The human IRS-1 protein contains the same Grb2 and Syp binding motifs of 897YVNI (underlined in Table 5, below and 1180YIDL (underlined in Table 5, below), respectively, as the rat IRS-1 protein. Mutants of hIRS-1 were constructed by substitution of a TAT codon (tyrosine) with a TTT codon (phenylalanine), in these motifs by use of oligonucleotide-directed mutagenesis using the following primers: (5'-GGGGGAATTGTCAATA-3' (SEQ ID NO:8) and 5'-GAATTTGTTAATATTG-3' (SEQ ID NO:9), respectively). The cDNAs of hIRS-1 (wild-type) and mutants (tyrosine 897-to-phenylalanine and tyrosine 1180-to-phenylalanine) were subcloned into the pBK-CMV expression vector and designated as hIRS-1-wt, 897F, ΔGrb2, 1180F, and ΔSyp.

TABLE 6

| Human IRS-1 amino acid sequence |            |             |            |            |            |      |
|---------------------------------|------------|-------------|------------|------------|------------|------|
| MASPPESDGF                      | SDVRKVGYLR | KPKSMHKRFF  | VLRAASEAGG | PARLEYEENE | KKWRHKSSAP | 61   |
| KRSIPLESCF                      | NINKRADSKN | KHLVALYTRD  | EHFAIAADSE | AEQDSWYQAL | LQLHNRAKGH | 121  |
| HDGAAALGAG                      | GGGGSCSGSS | GLGEAGEDLS  | YGDVPPGPAP | KEVWQVILKP | KGLGQTKNLI | 181  |
| GIYRLCLTSK                      | TISFVKLNSE | AAAVVLQLMN  | IRRCGHSENF | FFIEVGRSAV | TGPGEFWMQV | 241  |
| DDSVVAQNMH                      | ETILEAMRAM | SDEFRPRSKS  | QSSSNCSNPI | SVPLRRHHLN | NPPPSQVGLT | 301  |
| RRSRTESITA                      | TSPASVMVGK | PGSFRVRASS  | DGEGTMSRPA | SVDGSPVSPS | TNRTHAHRHR | 361  |
| GSARLHPPLN                      | HSRSIMPAS  | RCSPSATSPV  | SLSSSSTSGH | GSTDCLFPR  | RSSASVSGSP | 421  |
| SDGGFISSDE                      | YGSSPCDFRS | SFRSVTPDSL  | GHTPPARGE  | ELSNYICMG  | KGPSTLTAPN | 481  |
| GHYILSRGGN                      | GHRCTPGTGL | GTSPALAGDE  | AASAADLDNR | FRKRTHSAGT | SPTITHQKTP | 541  |
| SQSSVASIEE                      | YTEMMPAYPP | GGSGGRLPG   | HRHSAFVPTR | SYPEEGLEMH | PLERRGGHHR | 601  |
| PDSSTLHTDD                      | GYMPMSPGVA | PVPSPGRKGSG | DYMPMSPKSV | SAPQQIINPI | RRHPQRVDPN | 661  |
| GYMMSPSGG                       | CSPDIGGPGS | SSSSSSNAV   | SGTSYGKLWT | NGVGGHSHV  | LPHPKPPVES | 721  |
| SGGKLLPCTG                      | DYMNMPVGD  | SNTSSPSDCY  | YGPEDPQHKP | VLSYYSLPRS | FKHTQRPGE  | 781  |
| EEGARHQHLR                      | LSTSSGRLLY | AATADDSSSS  | TSSDSLGGGY | CGARLEPSLP | HPHHQVLQPH | 841  |
| LPRKVDTAAG                      | TNSRLARPTR | LSLGDPKAST  | LPRAREQQQQ | QQPLLHPPEP | KSPGEYVNI  | 901  |
| FGSDQSGYLS                      | GPVAFHSSPS | VRCPSQLQPA  | PREEETGTEE | YMKMDLGPR  | RAAWQESTGV | 961  |
| EMGRLGPAPP                      | GAASICRPTR | AVPSSRGDYM  | TMQMSCPRQS | YVDTSPAAPV | SYADMRTGIA | 1021 |
| AEVSLPRAT                       | MAAASSSSAA | SASPTGPQGA  | AELAAHSSLL | GGPQGPGGMS | AFTRVNLSPN | 1081 |
| RNQSAKVIRA                      | DPQGCRRRHS | SETFSSTPSA  | TRVGNTVPFG | AGAAVGGGGG | SSSSSEDKR  | 1141 |
| HSSASFENVW                      | LRPGELGGAP | KEPAKLCGAA  | GLENGLNYI  | DLDLVKDFKQ | CPQECTPEPQ | 1201 |
| PPPPPPPHQP                      | LGSGESSSTR | RSEEDLSAYA  | SISFQKQPED | RQ         |            |      |

(SEQ ID NO: 5; GENBANK Accession No. JS0670; pleckstrin domain spans residues 11-113, inclusive; Phosphate-binding residues include 46, 465, 551, 612, 632, 662, 732, 941, 989, or 1012 of SEQ ID NO: 5)

[0142]

TABLE 7

| Human IRS-1 cDNA |            |            |            |            |            |     |
|------------------|------------|------------|------------|------------|------------|-----|
| cggcggcgcg       | gtcggagggg | gccggcgcgc | agagccagac | gccgccgctt | gttttggttg | 61  |
| gggctctcgg       | caactctccg | aggaggagga | ggaggaggga | ggaggggaga | agtaactgca | 121 |
| gcggcagcgc       | cctcccagag | aacaggcgtc | ttccccgaac | ccttcccaaa | cctcccccat | 181 |
| ccccctctgc       | ccttgctccc | tcccctctc  | cccagccgcc | tggagcgagg | ggcagggatg | 241 |
| agtctgtccc       | tccggccggt | ccccagctgc | agtggtctgc | cgtatcggtt | tcgcatggaa | 301 |
| aagccacttt       | ctccaccgcg | cgagatgggc | cggatggggg | ctgcagagga | cgcgcccgcg | 361 |
| ggcggcggca       | gcagcagcag | cagcagcagc | agcaacagca | acagccgcag | cgcgcgggtc | 421 |
| tctgcgactg       | agctggtatt | tgggcggctg | gtggcggctg | ggacggttgg | ggggtgggag | 481 |
| gaggcgaagg       | aggagggaga | accccgtaga | acgttgggac | ttggcaaccc | gcctccccct | 541 |

TABLE 7-continued

| Human IRS-1 cDNA                                                  |      |  |  |  |  |
|-------------------------------------------------------------------|------|--|--|--|--|
| gccaaggat atttaatttg cctcgggaat cgctgcttcc agaggggaac tcaggaggga  | 601  |  |  |  |  |
| aggcgcgcg cgcgcgcgcg tcctggaggg gcaccgcagg gacccccgac tgcgcctcc   | 661  |  |  |  |  |
| ctgtgccgga ctccagccgg ggcgacgaga gatgcattct cgctccttcc tggtagcggc | 721  |  |  |  |  |
| ggcggctgag aggagacttg gctctcgag gatcggggct gccctcacc cggacgcact   | 781  |  |  |  |  |
| gcctccccg cggcgtgaag cgcggaaaa ctccggtcgg gctctctcct gggctcagca   | 841  |  |  |  |  |
| gctgctcct ccttcagctg cccctccccg gcgcgggggg cggcgtggat ttcagagtcg  | 901  |  |  |  |  |
| gggtttctgc tgctccagc cctgtttgca tgtgccgggc cgcggcgagg agcctccgc   | 961  |  |  |  |  |
| ccccaccgg ttgtttttcg gaggctccct ctgctcagcg ttggtggtgg cggtagcagc  | 1021 |  |  |  |  |
| atggcgagcc ctccggagag cgatggcttc tcggacgtgc gcaagggtgg ctacctgcgc | 1081 |  |  |  |  |
| aaaccaaga gcatgcacaa acgcttcttc gtactgcgcg cggccagcga ggtgggggc   | 1141 |  |  |  |  |
| ccggcgcgcc tcgagtacta cgagaacgag aagaagtggc ggcacaagtc gagcgcccc  | 1201 |  |  |  |  |
| aaacgctga tcccccttga gagctgcttc aacatcaaca agcgggctga ctccaagaac  | 1261 |  |  |  |  |
| aagcacctgg tggtctctta cccccggac gagcactttg ccacgcggc ggacagcgag   | 1321 |  |  |  |  |
| gccgagcaag acagctggta ccaggctctc ctacagctgc acaaccgtgc taagggccac | 1381 |  |  |  |  |
| cacgacggag ctgcggccct cggggcggga ggtggtgggg gcagctgcag cggcagctcc | 1441 |  |  |  |  |
| ggccttggtg aggtgggga ggaactgagc tacggtgacg tgccccagg acccgcatc    | 1501 |  |  |  |  |
| aaagaggtct ggcaagtgat cctgaagccc aagggcctgg gtcagacaaa gaacctgatt | 1561 |  |  |  |  |
| ggtatctacc gcctttgctt gaccagcaag accatcagct tcgtgaagct gaactcggag | 1621 |  |  |  |  |
| gcagcggccg tggtagctga gctgatgaac atcaggcgct gtggccactc ggaaaacttc | 1681 |  |  |  |  |
| ttcttcacg aggtgggccc ttctgccgtg acggggcccg gggagtcttg gatgcaggtg  | 1741 |  |  |  |  |
| gatgactctg tggtagccca gaacatgcac gagaccatcc tggaggccat gcgggccatg | 1801 |  |  |  |  |
| agtgatgagt tccgcctcgc cagcaagagc cagtcctcgt ccaactgctc taaccccatc | 1861 |  |  |  |  |
| agcgtcccc tgccgggca ccatctcaac aatccccgc ccagccaggt ggggctgacc    | 1921 |  |  |  |  |
| cgccgatcac gactgagag catcacgcc acctccccg ccagcatggt gggcgggaag    | 1981 |  |  |  |  |
| ccaggctcct tccgtgtccg cgcctccagt gacggcgaag gcaccatgct ccgcccagcc | 2041 |  |  |  |  |
| tcggtggagc gcagccctgt gagtccagc accaacagaa cccacgcca ccggcatcgg   | 2101 |  |  |  |  |
| ggcagcgcgc ggctgcaccc cccgctcaac cacagccgct ccattcccat gccggttcc  | 2161 |  |  |  |  |
| cgtgctcgc cttcgccac cagcccggtc agtctgtcgt ccagtagcac cagtggccat   | 2221 |  |  |  |  |
| ggctccacct cggattgtct ctccacgg cgatctagtg ctccggtgct tggttcccc    | 2281 |  |  |  |  |
| agcgtggcg gtttcatctc ctccgatgag tatggctcca gtccctgcga ttccggagt   | 2341 |  |  |  |  |
| tccttcgca gtgtcactcc ggattccctg ggccacacc caccagccc cggtaggag     | 2401 |  |  |  |  |
| gagctaagca actatatctg catgggtggc aaggggcctt ccacctgac cgcaccaac   | 2461 |  |  |  |  |
| ggtcactaca ttttgtctcg ggggtgcaat ggccaccgct gcacccagc aacaggcttg  | 2521 |  |  |  |  |
| ggcacgagtc cagccttgcc tggggatgaa gcagccagt ctgcagatct ggataatcg   | 2581 |  |  |  |  |
| ttccgaaaga gaactcactc gccaggcaca tccctacca ttaccacca gaagacccc    | 2641 |  |  |  |  |
| ttccagtcct cagtgccttc cattgaggag tacacagaga tgatgcctgc ctaccacca  | 2701 |  |  |  |  |
| ggagggtgca gtggaggccg actgcggga cacaggcact ccgccttcgt gccacccgc   | 2761 |  |  |  |  |

TABLE 7-continued

| Human IRS-1 cDNA |             |             |            |            |                  |
|------------------|-------------|-------------|------------|------------|------------------|
| tcctacccag       | aggaggggtct | ggaaatgcac  | cccttgagac | gtcggggggg | gcaccaccgc 2821  |
| ccagacagct       | ccacctcca   | cacggatgat  | ggctacatgc | ccatgtcccc | aggggtggcc 2881  |
| ccagtgccta       | gtggccgaaa  | gggcagtggg  | gactatatgc | ccatgagccc | caagagcgta 2941  |
| tctgccccac       | agcagatcat  | caatcccatc  | agacgccatc | cccagagagt | ggacccaat 3001   |
| ggctacatga       | tgatgtcccc  | cagcgggtggc | tgctctcctg | acattggagg | tggccccagc 3061  |
| agcagcagca       | gcagcagcaa  | cgccgtccct  | tccgggacca | gctatggaaa | gctgtggaca 3121  |
| aacggggtag       | ggggccacca  | ctctcatgtc  | ttgcctcacc | ccaaaccccc | agtggagagc 3181  |
| agcggtggtg       | agctcttacc  | ttgcacaggt  | gactacatga | acatgtcacc | agtgggggac 3241  |
| tccaacacca       | gcagcccctc  | cgactgctac  | tacggccctg | aggaccccca | gcacaagcca 3301  |
| gtcctctcct       | actactcatt  | gccaagatcc  | tttaagcaca | cccagcgcgc | cggggagccg 3361  |
| gaggaggggtg      | cccggcatca  | gcacctccgc  | ctttccacta | gctctggtcg | ccttctctat 3421  |
| gctgcaacag       | cagatgattc  | ttcctcttcc  | accagcagcg | acagcctggg | tgggggatac 3481  |
| tgccggggcta      | ggctggagcc  | cagccttcca  | catccccacc | atcaggttct | gcagcccat 3541   |
| ctgcctcgaa       | aggtggacac  | agctgctcag  | accaatagcc | gcctggcccg | gcccacgagg 3601  |
| ctgtccctgg       | gggatcccaa  | ggccagcacc  | ttacctcggg | cccagagca  | gcagcagcag 3661  |
| cagcagccct       | tgtgtcacc   | tccagagccc  | aagagcccg  | gggaatatgt | caatattgaa 3721  |
| tttgggagtg       | atcagtctgg  | ctacttgtct  | ggcccggtgg | ctttccacag | ctcaccttct 3781  |
| gtcaggtgtc       | catcccagct  | ccagccagct  | cccagagagg | aagagactgg | cactgaggag 3841  |
| tacatgaaga       | tggacctggg  | gccgggccgg  | agggcagcct | ggcaggagag | cactggggtc 3901  |
| gagatgggca       | gactggggcc  | tgcacctccc  | ggggctgcta | gcattttgag | gcctaccccg 3961  |
| gcagtgccta       | gcagccgggg  | tgactacatg  | accatgcaga | tgagttgtcc | ccgtcagagc 4021  |
| tacgtggaca       | cctcgccagc  | tgcccctgta  | agctatgctg | acatgcgaac | aggcattgct 4081  |
| gcagaggagg       | tgagcctgcc  | cagggccacc  | atggctgctg | cctcctcatc | ctcagcagcc 4141  |
| tctgttctcc       | cgactgggcc  | tcaaggggca  | gcagagctgg | ctgcccactc | gtccctgctg 4201  |
| ggggggccac       | aaggacctgg  | gggcatgagc  | gccttcaccc | gggtgaacct | cagtcctaac 4261  |
| cgcaaccaga       | gtgccaaagt  | gatccgtgca  | gaccacaaag | gggtgccggc | gaggcatagc 4321  |
| tccgagactt       | tctcctcaac  | accagtgccc  | accgggtggg | gcaacacagt | gccctttgga 4381  |
| gcggggggcag      | cagtaggggg  | cggtggcggt  | agcagcagca | gcagcgagga | tgtgaaacgc 4441  |
| cacagctctg       | cttcctttga  | gaatgtgtgg  | ctgaggcctg | gggagcttgg | gggagccccc 4501  |
| aaggagccag       | ccaaactgtg  | tggggctgct  | gggggtttgg | agaatggtct | taactacata 4561  |
| gacctggatt       | tggtaagga   | cttcaaacag  | tgccctcagg | agtgcacccc | tgaaccgcag 4621  |
| cctccccac        | ccccacccc   | tcatcaaccc  | ctgggcagcg | gtgagagcag | ctccacccgc 4681  |
| cgctcaagtg       | aggatttaag  | cgcctatgcc  | agcatcagtt | tccagaagca | gccagaggac 4741  |
| cgtcagtagc       | tcaactggac  | atcacagcag  | aatgaagacc | taaatgacct | cagcaaatcc 4801  |
| tcttctaact       | catgggtacc  | cagactctaa  | atatttcatg | attcacaact | aggacctcat 4861  |
| atcttctca        | tcagtagatg  | gtacgatgca  | tccatttcag | tttgtttact | ttatccaatc 4921  |
| ctcaggattt       | cattgactga  | actgcacgtt  | ctatatgttg | ccaagcgaaa | aaaaaaaaatg 4981 |

TABLE 7-continued

| Human IRS-1 cDNA                                                  |      |
|-------------------------------------------------------------------|------|
| cactgtgaca ccagaataat gagtctgcat aaacttcac ttcaacctta aggacttagc  | 5041 |
| tggccacagt gagctgatgt gcccaccacc gtgtcatgag agaatgggtt tactctcaat | 5101 |
| gcattttcaa gatacatttc atctgctgct gaaactgtgt acgacaaagc atcattgtaa | 5161 |
| attatttcat acaaaactgt tcacgttggg tggagagagt attaaatatt taacataggt | 5221 |
| tttgatttat atgtgtaatt ttttaaatga aaatgtaact tttcttacag cacatctttt | 5281 |
| ttttggatgt gggatggagg tatacaatgt tctgttgtaa agagtggagc aaatgcttaa | 5341 |
| aacaaggctt aaaagagtag aatagggtat gatccttggt ttaagattgt aattcagaaa | 5401 |
| acataatata agaatcatag tgccatagat ggttctcaat tgtatagtta tatttgctga | 5461 |
| tactatctct tgtcatataa acctgatgtt gagctgagtt ccttataaga attaatctta | 5521 |
| attttgtatt ttttcctgta agacaatagg ccatgttaat taaactgaag aaggatatat | 5581 |
| ttggctgggt gttttcaaat gtcagcttaa aattggtaat tgaatggaag caaaattata | 5641 |
| agaagaggaa attaaagtct tccattgcat gtattgtaaa cagaaggaga tgggtgattc | 5701 |
| cttcaattca aaagctctct ttggaatgaa caatgtgggc gtttgtaaat tctggaaatg | 5761 |
| tctttctatt cataataaac tagatactgt tgatctttta aaaaaaaaaa aaaaaaaaaa | 5821 |
| aaaaaaaa                                                          |      |

(SEQ ID NO: 6; GENBANK Accession No. NM 005544)

[0143] The double mutation of tyrosine 897 and 1180 was constructed by replacement of 3'-sequences coding 897F by the same region of 1180F using restriction enzymes NheI and EcoRI, and this construct was called 897F1180F or  $\Delta$ Grb2  $\Delta$ Syp. The expression plasmids were under control of a CMV promoter (hIRS-1-wt,  $\Delta$ Grb2,  $\Delta$ Syp,  $\Delta$ Grb2,  $\Delta$ Syp and pBK-CMV (mock) and linearized at the 3'-end of poly A signal sequences by MluI restriction enzymes followed by purification. A similar approach was used to change the tyrosine residue to phenylalanine at positions 613 and 942 to create the double PI3K mutant construct ( $\Delta$ PI3K). The hIRS-1 mutants have a FLAG epitope (DYKDDDDK (SEQ ID NO:6)+stop codon) added to the C-terminus by PCR.

This strategy allows to distinguish the mutant protein from "wild type" hIRS-1 in stable transfected cell lines. The mutants are used to define the link between the IRS signal transduction pathway and activation of HAAH as a downstream effector gene and identify compounds to inhibit transduction along the pathway to inhibit growth of tumors characterized by HAAH overexpression. Antibodies or other compounds which bind to phosphorylation sites or inhibit phosphorylation at those sites are used to inhibit signal transduction and thus proliferation of HAA-overexpressing tumors.

[0144] Other embodiments are within the following claims.

## SEQUENCE LISTING

&lt;160&gt; NUMBER OF SEQ ID NOS: 13

&lt;210&gt; SEQ ID NO 1

&lt;211&gt; LENGTH: 36

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Description of Artificial Sequence: Consensus EGF-like domain

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: VARIANT

&lt;222&gt; LOCATION: (2)..(8)

&lt;223&gt; OTHER INFORMATION: Wherein any Xaa may be any amino acid

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: VARIANT

&lt;222&gt; LOCATION: (10)..(13)

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<223> OTHER INFORMATION: Wherein Xaa is any amino acid.
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (15)..(24)
<223> OTHER INFORMATION: Wherein Xaa is any amino acid.
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (26)
<223> OTHER INFORMATION: Wherein Xaa is any amino acid.
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (28)..(35)
<223> OTHER INFORMATION: Wherein Xaa is any amino acid.

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<400> SEQUENCE: 1

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Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Cys Xaa Xaa
 1             5             10             15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys Xaa Xaa Xaa Xaa Xaa
      20             25             30

Xaa Xaa Xaa Cys
      35

```

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<210> SEQ ID NO 2
<211> LENGTH: 758
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 2

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Met Ala Gln Arg Lys Asn Ala Lys Ser Ser Gly Asn Ser Ser Ser Ser
 1             5             10             15

Gly Ser Gly Ser Gly Ser Thr Ser Ala Gly Ser Ser Ser Pro Gly Ala
      20             25             30

Arg Arg Glu Thr Lys His Gly Gly His Lys Asn Gly Arg Lys Gly Gly
      35             40             45

Leu Ser Gly Thr Ser Phe Phe Thr Trp Phe Met Val Ile Ala Leu Leu
      50             55             60

Gly Val Trp Thr Ser Val Ala Val Val Trp Phe Asp Leu Val Asp Tyr
      65             70             75             80

Glu Glu Val Leu Gly Lys Leu Gly Ile Tyr Asp Ala Asp Gly Asp Gly
      85             90             95

Asp Phe Asp Val Asp Asp Ala Lys Val Leu Leu Gly Leu Lys Glu Arg
      100            105            110

Ser Thr Ser Glu Pro Ala Val Pro Pro Glu Glu Ala Glu Pro His Thr
      115            120            125

Glu Pro Glu Glu Gln Val Pro Val Glu Ala Glu Pro Gln Asn Ile Glu
      130            135            140

Asp Glu Ala Lys Glu Gln Ile Gln Ser Leu Leu His Glu Met Val His
      145            150            155            160

Ala Glu His Val Glu Gly Glu Asp Leu Gln Gln Glu Asp Gly Pro Thr
      165            170            175

Gly Glu Pro Gln Gln Glu Asp Asp Glu Phe Leu Met Ala Thr Asp Val
      180            185            190

Asp Asp Arg Phe Glu Thr Leu Glu Pro Glu Val Ser His Glu Glu Thr
      195            200            205

Glu His Ser Tyr His Val Glu Glu Thr Val Ser Gln Asp Cys Asn Gln
      210            215            220

Asp Met Glu Glu Met Met Ser Glu Gln Glu Asn Pro Asp Ser Ser Glu

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| 225                        | 230                        | 235                    | 240     |
|----------------------------|----------------------------|------------------------|---------|
| Pro Val Val Glu Asp<br>245 | Glu Arg Leu His His<br>250 | Asp Thr Asp Asp<br>255 | Val Thr |
| Tyr Gln Val Tyr Glu<br>260 | Glu Gln Ala Val Tyr<br>265 | Glu Pro Leu Glu<br>270 | Asn Glu |
| Gly Ile Glu Ile Thr<br>275 | Glu Val Thr Ala Pro<br>280 | Pro Glu Asp Asn<br>285 | Pro Val |
| Glu Asp Ser Gln Val<br>290 | Ile Val Glu Glu Val<br>295 | Ser Ile Phe Pro<br>300 | Val Glu |
| Glu Gln Gln Glu Val<br>305 | Pro Pro Glu Thr Asn<br>310 | Arg Lys Thr Asp<br>315 | Asp Pro |
| Glu Gln Lys Ala Lys<br>325 | Val Lys Lys Lys Lys<br>330 | Pro Lys Leu Leu<br>335 | Asn Lys |
| Phe Asp Lys Thr Ile<br>340 | Lys Ala Glu Leu Asp<br>345 | Ala Ala Glu Lys<br>350 | Leu Arg |
| Lys Arg Gly Lys Ile<br>355 | Glu Glu Ala Val Asn<br>360 | Ala Phe Lys Glu<br>365 | Leu Val |
| Arg Lys Tyr Pro Gln<br>370 | Ser Pro Arg Ala Arg<br>375 | Tyr Gly Lys Ala<br>380 | Gln Cys |
| Glu Asp Asp Leu Ala<br>385 | Glu Lys Arg Arg Ser<br>390 | Asn Glu Val Leu<br>395 | Arg Gly |
| Ala Ile Glu Thr Tyr<br>405 | Gln Glu Val Ala Ser<br>410 | Leu Pro Asp Val<br>415 | Pro Ala |
| Asp Leu Leu Lys Leu<br>420 | Ser Leu Lys Arg Arg<br>425 | Ser Asp Arg Gln<br>430 | Gln Phe |
| Leu Gly His Met Arg<br>435 | Gly Ser Leu Leu Thr<br>440 | Leu Gln Arg Leu<br>445 | Val Gln |
| Leu Phe Pro Asn Asp<br>450 | Thr Ser Leu Lys Asn<br>455 | Asp Leu Gly Val<br>460 | Gly Tyr |
| Leu Leu Ile Gly Asp<br>465 | Asn Asp Asn Ala Lys<br>470 | Lys Lys Val Tyr<br>475 | Glu Glu |
| Leu Ser Val Thr Pro<br>485 | Asn Asp Gly Phe Ala<br>490 | Lys Val His Tyr<br>495 | Gly Phe |
| Ile Leu Lys Ala Gln<br>500 | Asn Lys Ile Ala Glu<br>505 | Ser Ile Pro Tyr<br>510 | Leu Lys |
| Glu Gly Ile Glu Ser<br>515 | Gly Asp Pro Gly Thr<br>520 | Asp Asp Gly Arg<br>525 | Phe Tyr |
| Phe His Leu Gly Asp<br>530 | Ala Met Gln Arg Val<br>535 | Gly Asn Lys Glu<br>540 | Ala Tyr |
| Lys Trp Tyr Glu Leu<br>545 | Gly His Lys Arg Gly<br>550 | His Phe Ala Ser<br>555 | Val Trp |
| Gln Arg Ser Leu Tyr<br>565 | Asn Val Asn Gly Leu<br>570 | Lys Ala Gln Pro<br>575 | Trp Trp |
| Thr Pro Lys Glu Thr<br>580 | Gly Tyr Thr Glu Leu<br>585 | Val Lys Ser Leu<br>590 | Glu Arg |
| Asn Trp Lys Leu Ile<br>595 | Arg Asp Glu Gly Leu<br>600 | Ala Val Met Asp<br>605 | Lys Ala |
| Lys Gly Leu Phe Leu<br>610 | Pro Glu Asp Glu Asn<br>615 | Leu Arg Glu Lys<br>620 | Gly Asp |
| Trp Ser Gln Phe Thr<br>625 | Leu Trp Gln Gln Gly<br>630 | Arg Arg Asn Glu<br>635 | Asn Ala |
|                            |                            |                        | 640     |

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Cys Lys Gly Ala Pro Lys Thr Cys Thr Leu Leu Glu Lys Phe Pro Glu  
                                 645                                650                                655  
 Thr Thr Gly Cys Arg Arg Gly Gln Ile Lys Tyr Ser Ile Met His Pro  
                                 660                                665                                670  
 Gly Thr His Val Trp Pro His Thr Gly Pro Thr Asn Cys Arg Leu Arg  
                                 675                                680                                685  
 Met His Leu Gly Leu Val Ile Pro Lys Glu Gly Cys Lys Ile Arg Cys  
                                 690                                695                                700  
 Ala Asn Glu Thr Arg Thr Trp Glu Glu Gly Lys Val Leu Ile Phe Asp  
                                 705                                710                                715                                720  
 Asp Ser Phe Glu His Glu Val Trp Gln Asp Ala Ser Ser Phe Arg Leu  
                                 725                                730                                735  
 Ile Phe Ile Val Asp Val Trp His Pro Glu Leu Thr Pro Gln Gln Arg  
                                 740                                745                                750  
 Arg Ser Leu Pro Ala Ile  
                                 755

&lt;210&gt; SEQ ID NO 3

&lt;211&gt; LENGTH: 2324

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 3

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cggaccgtgc aatggcccg cgtaagaatg ccaagagcag cggcaacagc agcagcagcg      60
gctccggcag cggtagcacg agtgcgggca gcagcagccc cggggcccg agagagacaa    120
agcatggagg acacaagaat gggaggaaag gcggaactctc gggaaactca ttcttcacgt    180
ggtttatggt gattgcattg ctgggcgtct ggacatctgt agctgtcgtt tggtttgatc    240
ttgttgacta tgaggaagtt ctaggaaaac taggaatcta tgatgctgat ggtgatggag    300
atthttgatgt ggatgatgcc aaagttttat taggacttaa agagagatct acttcagagc    360
cagcagtcgcc gccagaagag gctgagccac aactgagccc caggagcagc gttcctgtgg    420
aggcagaacc ccagaatatc gaagatgaag caaaagaaca aattcagtcc cttctccatg    480
aaatggtaca cgcagaacat gttgaggagg aagacttgca acaagaagat ggaccacacag    540
gagaaccaca acaagaggat gatgagtttc ttatggcgac tgatgtagat gatagatttg    600
agacctgga acctgaagta tctcatgaag aaaccgagca tagttaccac gtggaagaga    660
cagtttcaca agactgtaat caggatatgg aagagatgat gtctgagcag gaaaatccag    720
attccagtga accagtagta gaagatgaaa gattgcacca tgatacagat gatgtaacat    780
accaagtcta tgaggaacaa gcagtatatg aacctctaga aaatgaaggg atagaaatca    840
cagaagtaac tgctccccct gaggataatc ctgtagaaga ttcacaggta attgtagaag    900
aagtaagcat ttttcctgtg gaagaacagc aggaagtacc accagaaaca aatagaaaaa    960
cagatgatcc agaacaaaaa gcaaaagtta agaaaaagaa gcctaaactt ttaaataaat   1020
ttgataagac tattaagact gaacttgatg ctgcagaaaa actccgtaaa aggggaaaaa   1080
ttgaggaagc agtgaatgca tttaaagaac tagtacgcaa ataccctcag agtccacgag   1140
caagatatgg gaaggcgagc tgtgaggatg atttggctga gaagaggaga agtaaatgagg   1200
tgctacgtgg agccatcgag acctaccaag aggtggccag cctacctgat gtccctgcag   1260
acctgctgaa gctgagtttg aagcgtcgct cagacaggca acaatttcta ggtcatatga   1320

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-continued

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gagggtccct gcttaccctg cagagattag ttcaactatt tcccaatgat acttccttaa 1380
aaaaagacct tggcggtggga tacctcttga taggagataa tgacaatgca aagaaagttt 1440
atgaagaggt gctgagtgtg acacctaatt atggctttgc taaagtccat tatggcttca 1500
tcctgaaggc acagaacaaa attgctgaga gcatcccata tttaaaggaa ggaatagaat 1560
ccggagatcc tggcactgat gatgggagat tttatttcca cctgggggat gccatgcaga 1620
gggttgggaa caaagaggca tataagtggg atgagcttgg gcacaagaga ggacactttg 1680
catctgtctg gcaacgctca ctctacaatg tgaatggact gaaagcacag ccttggtgga 1740
cccaaaaaga aacgggctac acagagttag taaagtcttt agaagaaac tggaagttaa 1800
tccgagatga aggccttgca gtgatggata aagccaaagg tctcttcctg cctgaggatg 1860
aaaacctgag gaaaaaaggg gactggagcc agttcacgct gtggcagcaa ggaagaagaa 1920
atgaaaaatgc ctgcaaagga gctcctaaaa cctgtacctt actagaaaag tccccgaga 1980
caacaggatg cagaagagga cagatcaaat attccatcat gcaccccgagg actcacgtgt 2040
ggccgcacac agggcccaca aactgcaggc tccgaatgca cctgggcttg gtgattccca 2100
aggaaggctg caagattcga tgtgccaacg agaccaggac ctgggaggaa ggcaagggtg 2160
tcactcttga tgactccttt gagcacgagg tatggcagga tgcctcatct tccggctga 2220
tattcatcgt ggatgtgtg catccggaac tgacaccaca gcagagacgc agccttcag 2280
caatttagca tgaattcatg caagcttggg aaactctgga gaga 2324

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<210> SEQ ID NO 4
<211> LENGTH: 31
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: EGF-like
cysteine-rich repeat
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (2)..(5)
<223> OTHER INFORMATION: Wherein any Xaa may be any amino acid
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (7)..(8)
<223> OTHER INFORMATION: Wherein Xaa is any amino acid.
<220> FEATURE:
<221> NAME/KEY: VARIANT
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<223> OTHER INFORMATION: Wherein Xaa is any amino acid.
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<221> NAME/KEY: VARIANT
<222> LOCATION: (17)..(18)
<223> OTHER INFORMATION: Wherein Xaa is any amino acid.
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: Wherein Xaa is any amino acid.
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (29)
<223> OTHER INFORMATION: Wherein Xaa is any amino acid.
<400> SEQUENCE: 4

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Cys Asp Xaa Xaa Xaa Cys Xaa Xaa Lys Xaa Gly Asn Gly Xaa Cys Asp
  1             5             10             15

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-continued

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Xaa Xaa Cys Asn Asn Ala Ala Cys Xaa Xaa Asp Gly Xaa Asp Cys  
                   20                  25                  30

<210> SEQ ID NO 5  
 <211> LENGTH: 1242  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Met Ala Ser Pro Pro Glu Ser Asp Gly Phe Ser Asp Val Arg Lys Val  
   1                  5                  10                  15

Gly Tyr Leu Arg Lys Pro Lys Ser Met His Lys Arg Phe Phe Val Leu  
                   20                  25                  30

Arg Ala Ala Ser Glu Ala Gly Gly Pro Ala Arg Leu Glu Tyr Tyr Glu  
                   35                  40                  45

Asn Glu Lys Lys Trp Arg His Lys Ser Ser Ala Pro Lys Arg Ser Ile  
                   50                  55                  60

Pro Leu Glu Ser Cys Phe Asn Ile Asn Lys Arg Ala Asp Ser Lys Asn  
                   65                  70                  75                  80

Lys His Leu Val Ala Leu Tyr Thr Arg Asp Glu His Phe Ala Ile Ala  
                   85                  90                  95

Ala Asp Ser Glu Ala Glu Gln Asp Ser Trp Tyr Gln Ala Leu Leu Gln  
                   100                  105                  110

Leu His Asn Arg Ala Lys Gly His His Asp Gly Ala Ala Ala Leu Gly  
                   115                  120                  125

Ala Gly Gly Gly Gly Gly Ser Cys Ser Gly Ser Ser Gly Leu Gly Glu  
                   130                  135                  140

Ala Gly Glu Asp Leu Ser Tyr Gly Asp Val Pro Pro Gly Pro Ala Phe  
                   145                  150                  155                  160

Lys Glu Val Trp Gln Val Ile Leu Lys Pro Lys Gly Leu Gly Gln Thr  
                   165                  170                  175

Lys Asn Leu Ile Gly Ile Tyr Arg Leu Cys Leu Thr Ser Lys Thr Ile  
                   180                  185                  190

Ser Phe Val Lys Leu Asn Ser Glu Ala Ala Ala Val Val Leu Gln Leu  
                   195                  200                  205

Met Asn Ile Arg Arg Cys Gly His Ser Glu Asn Phe Phe Phe Ile Glu  
                   210                  215                  220

Val Gly Arg Ser Ala Val Thr Gly Pro Gly Glu Phe Trp Met Gln Val  
                   225                  230                  235                  240

Asp Asp Ser Val Val Ala Gln Asn Met His Glu Thr Ile Leu Glu Ala  
                   245                  250                  255

Met Arg Ala Met Ser Asp Glu Phe Arg Pro Arg Ser Lys Ser Gln Ser  
                   260                  265                  270

Ser Ser Asn Cys Ser Asn Pro Ile Ser Val Pro Leu Arg Arg His His  
                   275                  280                  285

Leu Asn Asn Pro Pro Pro Ser Gln Val Gly Leu Thr Arg Arg Ser Arg  
                   290                  295                  300

Thr Glu Ser Ile Thr Ala Thr Ser Pro Ala Ser Met Val Gly Gly Lys  
                   305                  310                  315                  320

Pro Gly Ser Phe Arg Val Arg Ala Ser Ser Asp Gly Glu Gly Thr Met  
                   325                  330                  335

Ser Arg Pro Ala Ser Val Asp Gly Ser Pro Val Ser Pro Ser Thr Asn

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| 340 |     |     |     |     | 345 |     |     |     |     | 350 |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Arg | Thr | His | Ala | His | Arg | His | Arg | Gly | Ser | Ala | Arg | Leu | His | Pro | Pro |
|     |     | 355 |     |     |     |     |     | 360 |     |     |     | 365 |     |     |     |
| Leu | Asn | His | Ser | Arg | Ser | Ile | Pro | Met | Pro | Ala | Ser | Arg | Cys | Ser | Pro |
|     | 370 |     |     |     |     | 375 |     |     |     |     | 380 |     |     |     |     |
| Ser | Ala | Thr | Ser | Pro | Val | Ser | Leu | Ser | Ser | Ser | Ser | Thr | Ser | Gly | His |
|     | 385 |     |     |     |     | 390 |     |     |     |     | 395 |     |     |     | 400 |
| Gly | Ser | Thr | Ser | Asp | Cys | Leu | Phe | Pro | Arg | Arg | Ser | Ser | Ala | Ser | Val |
|     |     |     |     | 405 |     |     |     |     | 410 |     |     |     |     | 415 |     |
| Ser | Gly | Ser | Pro | Ser | Asp | Gly | Gly | Phe | Ile | Ser | Ser | Asp | Glu | Tyr | Gly |
|     |     |     | 420 |     |     |     |     | 425 |     |     |     |     | 430 |     |     |
| Ser | Ser | Pro | Cys | Asp | Phe | Arg | Ser | Ser | Phe | Arg | Ser | Val | Thr | Pro | Asp |
|     |     | 435 |     |     |     |     | 440 |     |     |     |     | 445 |     |     |     |
| Ser | Leu | Gly | His | Thr | Pro | Pro | Ala | Arg | Gly | Glu | Glu | Glu | Leu | Ser | Asn |
|     | 450 |     |     |     |     | 455 |     |     |     |     | 460 |     |     |     |     |
| Tyr | Ile | Cys | Met | Gly | Gly | Lys | Gly | Pro | Ser | Thr | Leu | Thr | Ala | Pro | Asn |
|     | 465 |     |     |     |     | 470 |     |     |     |     | 475 |     |     |     | 480 |
| Gly | His | Tyr | Ile | Leu | Ser | Arg | Gly | Gly | Asn | Gly | His | Arg | Cys | Thr | Pro |
|     |     |     |     | 485 |     |     |     |     | 490 |     |     |     |     | 495 |     |
| Gly | Thr | Gly | Leu | Gly | Thr | Ser | Pro | Ala | Leu | Ala | Gly | Asp | Glu | Ala | Ala |
|     |     |     | 500 |     |     |     |     | 505 |     |     |     |     | 510 |     |     |
| Ser | Ala | Ala | Asp | Leu | Asp | Asn | Arg | Phe | Arg | Lys | Arg | Thr | His | Ser | Ala |
|     |     | 515 |     |     |     |     | 520 |     |     |     |     | 525 |     |     |     |
| Gly | Thr | Ser | Pro | Thr | Ile | Thr | His | Gln | Lys | Thr | Pro | Ser | Gln | Ser | Ser |
|     | 530 |     |     |     |     | 535 |     |     |     |     | 540 |     |     |     |     |
| Val | Ala | Ser | Ile | Glu | Glu | Tyr | Thr | Glu | Met | Met | Pro | Ala | Tyr | Pro | Pro |
|     | 545 |     |     |     |     | 550 |     |     |     |     | 555 |     |     |     | 560 |
| Gly | Gly | Gly | Ser | Gly | Gly | Arg | Leu | Pro | Gly | His | Arg | His | Ser | Ala | Phe |
|     |     |     |     | 565 |     |     |     |     | 570 |     |     |     |     | 575 |     |
| Val | Pro | Thr | Arg | Ser | Tyr | Pro | Glu | Glu | Gly | Leu | Glu | Met | His | Pro | Leu |
|     |     |     | 580 |     |     |     |     | 585 |     |     |     |     | 590 |     |     |
| Glu | Arg | Arg | Gly | Gly | His | His | Arg | Pro | Asp | Ser | Ser | Thr | Leu | His | Thr |
|     |     | 595 |     |     |     |     | 600 |     |     |     |     | 605 |     |     |     |
| Asp | Asp | Gly | Tyr | Met | Pro | Met | Ser | Pro | Gly | Val | Ala | Pro | Val | Pro | Ser |
|     | 610 |     |     |     |     | 615 |     |     |     |     | 620 |     |     |     |     |
| Gly | Arg | Lys | Gly | Ser | Gly | Asp | Tyr | Met | Pro | Met | Ser | Pro | Lys | Ser | Val |
|     | 625 |     |     |     |     | 630 |     |     |     |     | 635 |     |     |     | 640 |
| Ser | Ala | Pro | Gln | Gln | Ile | Ile | Asn | Pro | Ile | Arg | Arg | His | Pro | Gln | Arg |
|     |     |     |     | 645 |     |     |     |     | 650 |     |     |     |     | 655 |     |
| Val | Asp | Pro | Asn | Gly | Tyr | Met | Met | Met | Ser | Pro | Ser | Gly | Gly | Cys | Ser |
|     |     |     | 660 |     |     |     |     | 665 |     |     |     |     | 670 |     |     |
| Pro | Asp | Ile | Gly | Gly | Gly | Pro | Ser | Ser | Ser | Ser | Ser | Ser | Ser | Asn | Ala |
|     |     | 675 |     |     |     |     | 680 |     |     |     |     |     | 685 |     |     |
| Val | Pro | Ser | Gly | Thr | Ser | Tyr | Gly | Lys | Leu | Trp | Thr | Asn | Gly | Val | Gly |
|     | 690 |     |     |     |     |     | 695 |     |     |     |     | 700 |     |     |     |
| Gly | His | His | Ser | His | Val | Leu | Pro | His | Pro | Lys | Pro | Pro | Val | Glu | Ser |
|     | 705 |     |     |     |     | 710 |     |     |     |     | 715 |     |     |     | 720 |
| Ser | Gly | Gly | Lys | Leu | Leu | Pro | Cys | Thr | Gly | Asp | Tyr | Met | Asn | Met | Ser |
|     |     |     |     | 725 |     |     |     |     | 730 |     |     |     |     | 735 |     |
| Pro | Val | Gly | Asp | Ser | Asn | Thr | Ser | Ser | Pro | Ser | Asp | Cys | Tyr | Tyr | Gly |
|     |     |     | 740 |     |     |     |     | 745 |     |     |     |     | 750 |     |     |

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|     |      |      |      |     |      |      |      |      |     |      |      |      |      |      |      |
|-----|------|------|------|-----|------|------|------|------|-----|------|------|------|------|------|------|
| Pro | Glu  | Asp  | Pro  | Gln | His  | Lys  | Pro  | Val  | Leu | Ser  | Tyr  | Tyr  | Ser  | Leu  | Pro  |
|     | 755  |      |      |     |      |      | 760  |      |     |      |      | 765  |      |      |      |
| Arg | Ser  | Phe  | Lys  | His | Thr  | Gln  | Arg  | Pro  | Gly | Glu  | Pro  | Glu  | Glu  | Gly  | Ala  |
|     | 770  |      |      |     |      | 775  |      |      |     |      | 780  |      |      |      |      |
| Arg | His  | Gln  | His  | Leu | Arg  | Leu  | Ser  | Thr  | Ser | Ser  | Gly  | Arg  | Leu  | Leu  | Tyr  |
|     | 785  |      |      |     | 790  |      |      |      |     | 795  |      |      |      |      | 800  |
| Ala | Ala  | Thr  | Ala  | Asp | Asp  | Ser  | Ser  | Ser  | Ser | Thr  | Ser  | Ser  | Asp  | Ser  | Leu  |
|     |      |      |      | 805 |      |      |      |      |     | 810  |      |      |      | 815  |      |
| Gly | Gly  | Gly  | Tyr  | Cys | Gly  | Ala  | Arg  | Leu  | Glu | Pro  | Ser  | Leu  | Pro  | His  | Pro  |
|     |      |      | 820  |     |      |      |      | 825  |     |      |      |      | 830  |      |      |
| His | His  | Gln  | Val  | Leu | Gln  | Pro  | His  | Leu  | Pro | Arg  | Lys  | Val  | Asp  | Thr  | Ala  |
|     | 835  |      |      |     |      |      | 840  |      |     |      |      | 845  |      |      |      |
| Ala | Gln  | Thr  | Asn  | Ser | Arg  | Leu  | Ala  | Arg  | Pro | Thr  | Arg  | Leu  | Ser  | Leu  | Gly  |
|     | 850  |      |      |     |      | 855  |      |      |     |      | 860  |      |      |      |      |
| Asp | Pro  | Lys  | Ala  | Ser | Thr  | Leu  | Pro  | Arg  | Ala | Arg  | Glu  | Gln  | Gln  | Gln  | Gln  |
|     | 865  |      |      |     | 870  |      |      |      |     | 875  |      |      |      |      | 880  |
| Gln | Gln  | Pro  | Leu  | Leu | His  | Pro  | Pro  | Glu  | Pro | Lys  | Ser  | Pro  | Gly  | Glu  | Tyr  |
|     |      |      | 885  |     |      |      |      | 890  |     |      |      |      |      | 895  |      |
| Val | Asn  | Ile  | Glu  | Phe | Gly  | Ser  | Asp  | Gln  | Ser | Gly  | Tyr  | Leu  | Ser  | Gly  | Pro  |
|     |      | 900  |      |     |      |      |      | 905  |     |      |      |      | 910  |      |      |
| Val | Ala  | Phe  | His  | Ser | Ser  | Pro  | Ser  | Val  | Arg | Cys  | Pro  | Ser  | Gln  | Leu  | Gln  |
|     |      | 915  |      |     |      |      | 920  |      |     |      |      | 925  |      |      |      |
| Pro | Ala  | Pro  | Arg  | Glu | Glu  | Glu  | Thr  | Gly  | Thr | Glu  | Glu  | Tyr  | Met  | Lys  | Met  |
|     | 930  |      |      |     |      | 935  |      |      |     |      |      | 940  |      |      |      |
| Asp | Leu  | Gly  | Pro  | Gly | Arg  | Arg  | Ala  | Ala  | Trp | Gln  | Glu  | Ser  | Thr  | Gly  | Val  |
|     | 945  |      |      |     | 950  |      |      |      |     | 955  |      |      |      |      | 960  |
| Glu | Met  | Gly  | Arg  | Leu | Gly  | Pro  | Ala  | Pro  | Pro | Gly  | Ala  | Ala  | Ser  | Ile  | Cys  |
|     |      |      | 965  |     |      |      |      | 970  |     |      |      |      |      | 975  |      |
| Arg | Pro  | Thr  | Arg  | Ala | Val  | Pro  | Ser  | Ser  | Arg | Gly  | Asp  | Tyr  | Met  | Thr  | Met  |
|     |      | 980  |      |     |      |      |      | 985  |     |      |      |      | 990  |      |      |
| Gln | Met  | Ser  | Cys  | Pro | Arg  | Gln  | Ser  | Tyr  | Val | Asp  | Thr  | Ser  | Pro  | Ala  | Ala  |
|     |      | 995  |      |     |      |      | 1000 |      |     |      |      | 1005 |      |      |      |
| Pro | Val  | Ser  | Tyr  | Ala | Asp  | Met  | Arg  | Thr  | Gly | Ile  | Ala  | Ala  | Glu  | Glu  | Val  |
|     | 1010 |      |      |     |      | 1015 |      |      |     |      | 1020 |      |      |      |      |
| Ser | Leu  | Pro  | Arg  | Ala | Thr  | Met  | Ala  | Ala  | Ala | Ser  | Ser  | Ser  | Ser  | Ala  | Ala  |
|     | 1025 |      |      |     | 1030 |      |      |      |     | 1035 |      |      |      |      | 1040 |
| Ser | Ala  | Ser  | Pro  | Thr | Gly  | Pro  | Gln  | Gly  | Ala | Ala  | Glu  | Leu  | Ala  | Ala  | His  |
|     |      |      | 1045 |     |      |      |      | 1050 |     |      |      |      |      | 1055 |      |
| Ser | Ser  | Leu  | Leu  | Gly | Gly  | Pro  | Gln  | Gly  | Pro | Gly  | Gly  | Met  | Ser  | Ala  | Phe  |
|     |      | 1060 |      |     |      |      |      | 1065 |     |      |      |      | 1070 |      |      |
| Thr | Arg  | Val  | Asn  | Leu | Ser  | Pro  | Asn  | Arg  | Asn | Gln  | Ser  | Ala  | Lys  | Val  | Ile  |
|     | 1075 |      |      |     |      |      | 1080 |      |     |      |      | 1085 |      |      |      |
| Arg | Ala  | Asp  | Pro  | Gln | Gly  | Cys  | Arg  | Arg  | Arg | His  | Ser  | Ser  | Glu  | Thr  | Phe  |
|     | 1090 |      |      |     |      | 1095 |      |      |     |      | 1100 |      |      |      |      |
| Ser | Ser  | Thr  | Pro  | Ser | Ala  | Thr  | Arg  | Val  | Gly | Asn  | Thr  | Val  | Pro  | Phe  | Gly  |
|     | 1105 |      |      |     | 1110 |      |      |      |     | 1115 |      |      |      |      | 1120 |
| Ala | Gly  | Ala  | Ala  | Val | Gly  | Gly  | Gly  | Gly  | Gly | Ser  | Ser  | Ser  | Ser  | Ser  | Glu  |
|     |      |      | 1125 |     |      |      |      | 1130 |     |      |      |      |      | 1135 |      |
| Asp | Val  | Lys  | Arg  | His | Ser  | Ser  | Ala  | Ser  | Phe | Glu  | Asn  | Val  | Trp  | Leu  | Arg  |
|     |      | 1140 |      |     |      |      |      | 1145 |     |      |      |      | 1150 |      |      |

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Pro Gly Glu Leu Gly Gly Ala Pro Lys Glu Pro Ala Lys Leu Cys Gly  
 1155 1160 1165

Ala Ala Gly Gly Leu Glu Asn Gly Leu Asn Tyr Ile Asp Leu Asp Leu  
 1170 1175 1180

Val Lys Asp Phe Lys Gln Cys Pro Gln Glu Cys Thr Pro Glu Pro Gln  
 1185 1190 1195 1200

Pro Pro Pro Pro Pro Pro Pro His Gln Pro Leu Gly Ser Gly Glu Ser  
 1205 1210 1215

Ser Ser Thr Arg Arg Ser Ser Glu Asp Leu Ser Ala Tyr Ala Ser Ile  
 1220 1225 1230

Ser Phe Gln Lys Gln Pro Glu Asp Arg Gln  
 1235 1240

&lt;210&gt; SEQ ID NO 6

&lt;211&gt; LENGTH: 5828

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 6

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gggctctcgg caactctccg aggaggagga ggaggagga ggaggggaga agtaactgca      120
gcggcagcgc cctcccagg aacaggcgct tccccgaac ccttccaaa cctcccccat      180
ccctctcgc ccttgctccc tcccctcctc ccagccgcc tggagcgagg gccagggatg      240
agtctgtccc tccggccggt cccagctgc agtggtgcc cgttatcgtt tcgcatggaa      300
aagccacttt ctccaccgc cgagatggg cgggatggg ctgcagagga cgcgcccgcg      360
ggcggcggca gcagcagcag cagcagcagc agcaacagca acagccgag cgcgcgggctc      420
tgtgcgactg agctggtatt tgggcggctg gtggcggctg ggacgggttg ggggtgggag      480
gaggcgaagg aggagggaga acccctgca acgttgggac ttggcaacct gcctccccct      540
gcccaaggat atttaatttg cctcggaat cgctgcttcc agaggggaac tcaggaggga      600
aggcgcgcg gcgcgcgcgc tcctggagg gcaccgcagg gacccccgac tgcgcctcc      660
ctgtgccgga ctccagccgg ggcgacgaga gatgcattt cgctccttcc tggtgccggc      720
ggcggtgtag aggagacttg gctctcgag gatcggggct gccctcacc cggacgcact      780
gcctccccgc cggcgtgaag cgcgcgaaaa ctccggtcgg gctctctcct gggctcagca      840
gtgctgctct ccttcagctg cccctccccg gcgcgggggg cggcgtggat ttcagagtcg      900
gggtttctgc tgctccagc cctgtttgca tgtgccgggc cgcggcgagg agcctccgcc      960
ccccaccgg ttgtttttcg gagcctccct ctgctcagcg ttggtggttg cgtggcagc     1020
atggcgagcc ctccggagag cgatggcttc tcggacgtgc gcaaggtggg ctacctgcgc     1080
aaaccaaga gcatgcacaa acgcttcttc gtactgcgcg cggccagcga ggctgggggc     1140
ccggcgcgcc tcgagtacta cgagaacgag aagaagtggc ggcacaagtc gagcgcccc     1200
aaacgctcga tcccccttga gagctgcttc aacatcaaca agcgggctga ctccaagaac     1260
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gccgagcaag acagctggta ccaggctctc ctacagctgc acaaccgtgc taagggccac     1380
cacgagggag ctgcggccct cggggcggga ggtggtgggg gcagctgcag cgcagctcc     1440
ggccttggtg aggctgggga ggacttgagc tacggtgacg tgccccagc acccgattc     1500

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|             |             |            |            |            |             |      |
|-------------|-------------|------------|------------|------------|-------------|------|
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| ggtatctacc  | gcctttgcct  | gaccagcaag | accatcagct | tcgtgaagct | gaactcggag  | 1620 |
| gcagcggccg  | tgggtgctga  | gctgatgaac | atcaggcgct | gtggccactc | ggaaaacttc  | 1680 |
| ttcttcacgt  | aggtgggccc  | ttctgcccgt | acggggcccc | gggagttctg | gatgcagggt  | 1740 |
| gatgactctg  | tgggtggccc  | gaacatgcac | gagaccatcc | tggaggccat | gcggggccatg | 1800 |
| agtgatgagt  | tccgcctctg  | cagcaagagc | cagtcctcgt | ccaactgctc | taaccccatc  | 1860 |
| agcgtccccc  | tgcgcggcca  | ccatctcaac | aatccccccg | ccagccaggt | ggggctgacc  | 1920 |
| cgcgatcac   | gactgagag   | catcaccgcc | acctccccgg | ccagcatggg | ggcggggaag  | 1980 |
| ccaggctcct  | tccgtgtccg  | cgcctccagt | gacggcgaag | gcaccatgtc | ccgccccagcc | 2040 |
| tcggtggacg  | gcagccctgt  | gagtcaccgc | accaacagaa | cccacgcccc | ccggcatcgg  | 2100 |
| ggcagcggcc  | ggctgcaccc  | cccgtcaac  | cacagccgct | ccatcccat  | gccgggttcc  | 2160 |
| cgtgtctcgc  | cttcggccac  | cagcccggtc | agtctgtcgt | ccagtagcac | cagtggccat  | 2220 |
| ggctccacct  | cggattgtct  | cttcccacgg | cgatctagt  | cttcgggtgc | tggttccccc  | 2280 |
| agcgatggcg  | gtttcatctc  | ctcgatgag  | tatggctcca | gtccctgcga | ttcccgaggt  | 2340 |
| tccttcgcga  | gtgtcactcc  | ggattccctg | ggccacaccc | caccagcccg | cggtagggag  | 2400 |
| gagctaagca  | actatatctg  | catgggtggc | aaggggccct | ccaccctgac | cgcgcccaac  | 2460 |
| ggcactaca   | ttttgtctcg  | gggtggcaat | ggccaccgct | gcacccaggg | aacaggcttg  | 2520 |
| ggcacgagtc  | cagccttggc  | tggggatgaa | gcagccagtg | ctgcagatct | ggataatcgg  | 2580 |
| ttccgaaaga  | gaactcactc  | ggcaggcaca | tcccctacca | ttaccacca  | gaagaccccg  | 2640 |
| tcccagtcct  | cagtggcttc  | cattgaggag | tacacagaga | tgatgcctgc | ctaccacca   | 2700 |
| ggaggtggca  | gtggaggccg  | actgcggga  | cacaggcact | ccgccttcgt | gccacccgc   | 2760 |
| tcctaccacg  | aggaggggtct | ggaaatgcac | cccttgagc  | gtcggggggg | gcaccaccgc  | 2820 |
| ccagacagct  | ccaccctcca  | cacggatgat | ggctacatgc | ccatgtcccc | aggggtggcc  | 2880 |
| ccagtgccca  | gtggccgaaa  | gggcagtgga | gactatatgc | ccatgagccc | caagagcgta  | 2940 |
| tctgccccac  | agcagatcat  | caatcccatc | agacgccatc | cccagagagt | ggaccccaat  | 3000 |
| ggctacatga  | tgatgtcccc  | cagcgggtgg | tgtctctcct | acattggagg | tggccccagc  | 3060 |
| agcagcagca  | gcagcagcaa  | cgcctccct  | tccgggacca | gctatggaaa | gctgtggaca  | 3120 |
| aacggggtag  | ggggccacca  | ctctcatgtc | ttgcctcacc | ccaaaccccc | agtggagagc  | 3180 |
| agcgggtgta  | agctcttacc  | ttgcacaggt | gactacatga | acatgtcacc | agtgggggac  | 3240 |
| tccaacacca  | gcagccccct  | cgaactgtac | tacggccctg | aggaccccca | gcacaagcca  | 3300 |
| gtcctctcct  | actactcatt  | gccaagatcc | tttaagcaca | cccagcggcc | cggggagccg  | 3360 |
| gaggaggggtg | cccggcatca  | gcacctccgc | ctttccacta | gctctgggtc | ccttctctat  | 3420 |
| gtgcaacag   | cagatgattc  | ttcctcttcc | accagcagcg | acagcctggg | tgggggatac  | 3480 |
| tgcggggcta  | ggctggagcc  | cagccttcca | catccccacc | atcaggttct | gcagcccat   | 3540 |
| ctgcctcgaa  | aggtggacac  | agctgctcag | accaatagcc | gcctggcccc | gcccacgagg  | 3600 |
| ctgtccctgg  | gggatcccaa  | ggccagcacc | ttacctcggg | cccagagaca | gcagcagcag  | 3660 |
| cagcagccct  | tgtctgaccc  | tccagagccc | aagagcccg  | gggaatatgt | caatattgaa  | 3720 |
| tttgggagtg  | atcagctctg  | ctacttgtct | ggcccggtgg | ctttccacag | ctcaccttct  | 3780 |

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|                                                                    |      |
|--------------------------------------------------------------------|------|
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| tacatgaaga tggacctggg gccgggccgg agggcagcct ggcaggagag cactggggtc  | 3900 |
| gagatgggca gactggggcc tgcacctccc ggggctgcta gcatttgtag gcctacccgg  | 3960 |
| gcagtgcccc gcagccgggg tgactacatg accatgcaga tgagttgtcc ccgtcagagc  | 4020 |
| tacgtggaca cctcgcagc tgcacctgta agctatgctg acatgcgaac aggcattgct   | 4080 |
| gcagaggagg tagcctgcc cagggccacc atggctgctg cctcctcatc ctacagagcc   | 4140 |
| tctgttcccc cgactgggcc tcaaggggca gcagagctgg ctgccactc gtccctgctg   | 4200 |
| ggggggccac aaggacctgg gggcatgagc gccttcaccc gggtgaaact cagtcctaac  | 4260 |
| cgcaaccaga gtgccaaagt gatccgtgca gaccacaaag ggtgccggcg gaggcatagc  | 4320 |
| tccgagactt tctcctcaac acccagtgcc acccggtgg gcaacacagt gccctttgga   | 4380 |
| gcggggggcag cagtaggggg cgggtggcgt agcagcagca gcagcgagga tgtgaaacgc | 4440 |
| cacagctctg ctctctttga gaatgtgtgg ctgaggcctg gggagcttgg gggagcccc   | 4500 |
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| gacctggatt tgggtcaagga cttcaaacag tgccctcagg agtgcacccc tgaaccgcag | 4620 |
| cctccccac ccccccccc tcatcaaccc ctgggcagcg gtgagagcag ctccacccgc    | 4680 |
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| cgtcagtagc tcaactggac atcacagcag aatgaagacc taaatgacct cagcaaatcc  | 4800 |
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| tttgatttat atgtgtaatt ttttaaatga aaatgtaact tttcttacag cacatctttt  | 5280 |
| ttttggatgt gggatggagg tatacaatgt tctgttgtaa agagtggagc aaatgcttaa  | 5340 |
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| acataatata agaataatag tgccatagat ggttctcaat tgtatagtta tatttgctga  | 5460 |
| tactatctct tgcatataa acctgatgtt gagctgagtt ccttataaga attaatctta   | 5520 |
| attttgtatt ttttctgta agacaatagg ccatgttaat taaactgaag aaggatatat   | 5580 |
| ttggctgggt gttttcaaat gtcagcttaa aattggtaat tgaatggaag caaaattata  | 5640 |
| agaagaggaa attaaagtct tccattgcat gtattgtaaa cagaaggaga tgggtgattc  | 5700 |
| cttcaattca aaagctctct ttggaatgaa caatgtgggc gtttgtaaat tctggaaatg  | 5760 |
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| aaaaaaaa                                                           | 5828 |

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&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence: FLAG epitope

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Asp Tyr Lys Asp Asp Asp Asp Lys  
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<210> SEQ ID NO 8

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<223> OTHER INFORMATION: Description of Artificial Sequence: Mutagenesis primer

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<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Location (-1) oligonucleotide

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<220> FEATURE:

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<210> SEQ ID NO 13

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<212> TYPE: DNA
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What is claimed is:

1. A method of inhibiting tumor growth in a mammal comprising administering to said mammal a compound which inhibits expression of HAAH.

2. The method of claim 1, wherein said compound is a HAAH antisense nucleic acid.

3. The method of claim 2, wherein said HAAH antisense nucleic acid is 10-50 nucleotides in length.

4. The method of claim 2, wherein said the sequence of said HAAH antisense nucleic acid is complementary to a nucleic acid sequence is the 5' untranslated region of a HAAH gene.

5. The method of claim 2, wherein the sequence of said HAAH antisense nucleic acid comprises 5'CAT TCT TAC GCT GGG CCA TT 3' (SEQ ID NO:10).

6. The method of claim 2, wherein the sequence of said HAAH antisense nucleic acid comprises 5' TTA CGC TGG GCC ATT GCA CG 3' (SEQ ID NO:11)

7. The method of claim 2, wherein the sequence of said HAAH antisense nucleic acid comprises 5' CTG GGC CAT TGC ACG GTC CG 3' (SEQ ID NO:12).

8. The method of claim 1, wherein said compound is a ribozyme.

9. The method of claim 1, wherein said tumor is derived from endodermal tissue.

10. The method of claim 1, wherein said tumor is selected from the group consisting of colon cancer, breast cancer, pancreatic cancer, liver cancer, and cancer of the bile ducts.

11. The method of claim 10, wherein said tumor is a CNS tumor.

12. An HAAH antisense nucleic acid, wherein said nucleic acid comprises a sequence which is complementary to a sequence of a HAAH gene.

13. An HAAH antisense nucleic acid, wherein said nucleic acid comprises a sequence which is complementary to a noncoding sequence of a HAAH gene.

14. An HAAH antisense nucleic acid, wherein said nucleic acid comprises a sequence which is complementary to a sequence in the 5' untranslated region of a HAAH gene.

15. The nucleic acid of claim 14, wherein the sequence of said HAAH antisense nucleic acid comprises 5'CAT TCT TAC GCT GGG CCA TT 3' (SEQ ID NO:10).

16. The nucleic acid of claim, 14, wherein the sequence of said HAAH antisense nucleic acid comprises 5' TTA CGC TGG GCC ATT GCA CG 3' (SEQ ID NO:11)

17. The nucleic acid of claim, 14, wherein the sequence of said HAAH antisense nucleic acid comprises 5' CTG GGC CAT TGC ACG GTC CG 3' (SEQ ID NO:12).

18. A method of conferring an immune response to a tumor cell in a mammal, comprising administering to said mammal an antibody which binds to aspartyl (asparaginy) beta hydroxylase (HAAH).

19. The method of claim 18, wherein said tumor cell is a brain tumor cell.

20. The method of claim 19, wherein said brain tumor cell is selected from the group consisting of a glioma, a glioblastoma, an astrocytoma, and a hemangioma.

21. The method of claim 18, wherein said tumor cell is a pancreatic carcinoma cell.

22. The method of claim 18, wherein said antibody binds to an extracellular domain of HAAH.

23. The method of claim 18, wherein said antibody binds to a catalytic domain of HAAH.

24. The method of claim 23, wherein said catalytic domain comprises amino acids 660-700 of SEQ ID NO:2.

25. The method of claim 18, wherein said antibody is FB50 or a fragment thereof.

26. The method of claim 18, wherein said antibody is selected from the group consisting of FB50, 86A, 5C7 and 19B.

27. The method of claim 18 wherein said antibody is a mixture of one or more antibodies selected from the group consisting of FB50, 86A, 5C7 and 19B.

28. The method of claim 18, wherein said antibody is a high affinity single chain antibody.

29. A method of inhibiting tumor growth in a mammal, comprising administering to said mammal an HAAH-binding antibody conjugated to a cytotoxic agent.

30. A method of inducing an HAAH-specific immune response in a mammal, comprising administering to said mammal an HAAH polypeptide.

31. The method of claim 30, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:2.

32. The method of claim 30, wherein said polypeptide comprises an extracellular domain of HAAH and lacks an intracellular domain of HAAH.

33. The method of claim 30, wherein said polypeptide comprises a catalytic domain of HAAH.

34. The method of claim 33, wherein said polypeptide comprises amino acids 650-700 of SEQ ID NO:2.

35. The method of claim 30, further comprising administering an adjuvant composition.

36. A method of inducing an HAAH-specific immune response in a mammal, comprising administering to said mammal a polynucleotide composition encoding an HAAH polypeptide, or a degenerate variant of said polynucleotide.

37. The method of claim 36, wherein said composition comprises a transfection-enhancing agent.

38. The method of claim 36, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:2.

39. The method of claim 36, wherein said polypeptide comprises an extracellular domain of HAAH and lacks an intracellular domain of HAAH.

40. The method of claim 36, wherein said polypeptide comprises a catalytic domain of HAAH.

41. The method of claim 40, wherein said polypeptide comprises amino acids 650-700 of SEQ ID NO:2.

42. A method for diagnosing a neoplasm in a mammal, comprising contacting a tissue of said mammal with a detectably-labeled antibody which binds to HAAH, wherein an increase in the level of antibody binding at a tissue site compared to the level of binding to a normal nonneoplastic tissue indicates the presence of a neoplasm at said tissue site.

43. The method of claim 42, wherein said antibody is labeled with a radioactive compound.

44. The method of claim 43, wherein said radioactive compound is selected from the group consisting of  $^{125}\text{I}$ ,  $^{99}\text{Tc}$ .

45. The method of claim 42, wherein said antibody is labeled with  $\text{Gd}^{+++}$  or  $\text{Fe}^{++}$ .

46. The method of claim 42, wherein said antibody is labeled with a colorimetric agent.

47. The method of claim 42, wherein said tissue is a lymphoid tissue.

48. A fragment of HAAH comprising an extracellular domain and lacking a cytoplasmic domain of said HAAH.

49. A fragment of HAAH, wherein said fragment lacks residues 660-758 of SEQ ID NO:2.

50. A fragment of HAAH, wherein said fragment lacks residues 679-697 of SEQ ID NO:2.

51. A fragment of HAAH, wherein said fragment lacks at least one residue of SEQ ID NO:2, wherein said residue is selected from the group consisting of residue 661, 662, 663, 670, 671, 672, and 673.

52. A fragment of HAAH, wherein said fragment lacks enzymatic activity.

53. A fragment of HAAH, wherein said fragment lacks an alpha-ketoglutarate binding domain and an EGF-like domain.

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