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BACKGROUND OF THE INVENTION

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and is the fifth most common cancer in humans worldwide. HCC also is the fourth leading cause of cancer-related death (Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94:153-156). In 1990, the World Health Organization estimated that there were about 430,000 new cases of liver cancer worldwide, and that a similar number of patients died that year as a result of this disease.

The pathogenesis of HCC has been associated with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, as well as cirrhosis-inducing conditions of liver (Bruix J, et al. J Hepatol 35:421-430, 2001; Bruix J, et al. Cancer Cell 5:215-219, 2004). Accordingly, the incidence of HCC is highest in east Asian countries, such as China, Hong Kong, Taiwan, Korea, and Japan, where HBV and HCV infections are most prevalent (Bruix J, et al. Cancer Cell 5:215-219, 2004; Haskell CM. Chapter 46 Liver: Natural History, Diagnosis and Staging in "Cancer Treatment" 5th edition, W. B, Saunders Company, Philadelphia, editors:Haskell CM & Berek JS). However, the incidence of HCC in western countries is steadily increasing (Parkin DM, et al. Int J Cancer 94; 153-156, 2001). Over the past decade in the United States, HCC displayed the second highest increase in incidence, and the highest increase in death rate, of all cancers (Ann Int Med 139:817-823, 2003). Thus, in the United States and throughout the world, HCC is a major cause of mortality and morbidity, and a significant economic burden due to hospital costs and loss of work by people with HCC.

Successful control of HCC requires correct diagnosis of the disease at an early stage of disease progression. However, distinguishing small HCC tumors from other malignant or non-malignant liver diseases, including metastatic tumors, cholangiocarcinoma, focal nodular hyperplasia, dysplastic and regenerating liver nodules, using current techniques, such as imaging studies, needle core biopsy and/or fine needle aspiration, has proven to be challenging (Ferrell LD, et al. Am J Surg Pathol 17:1113-1123, 1993; Horigome H, et al. Hepato-Gatroenterology 47:1659-1662, 2000; Kalar S, et al. Arch Pathol Lab Med 131:1648-1654, 2007; Seki S, et al. Clin Cancer Res 6:3460-3473, 2000). Moreover, attempts to treat HCC therapeutically have been largely unsuccessful (Bruix J, et al. J Hepatol 35:421-430, 2001; Bruix J, et al. Cancer Cell 5:215-219, 2004; Haskell CM. Chapter 46 Liver: Natural History, Diagnosis and Staging in "Cancer Treatment" 5th edition, W. B, Saunders Company, Philadelphia, editors:Haskell CM & Berek JS; Szklaruk J, et al. AJR 180:441-453, 2003). As a result, despite active therapy, the 5-year survival rate of patients with HCC in the U.S. is only 10.5%, which is second in magnitude only to pancreatic cancer (ACS Cancer Facts & Figures (2007)). Thus, there is an urgent need to identify a more reliable marker to differentiate HCC from other liver pathologies and facilitate early detection of this disease. In addition, there is an urgent need to develop new and more-effective therapeutic agents for the treatment of HCC.

WO 03/024392 discloses that tumor-associated vasculature in hepatocellular carcinomas was strongly positive for *in situ* hybridization of TAT215, corresponding to present SEQ ID NO: 23.

SUMMARY OF THE INVENTION

The subject matter of the invention is defined in the appended claims.

The invention relates to a method of diagnosing a hepatocellular carcinoma (HCC) in a subject (*e.g.*, a human), comprising detecting the level of PLVAP protein in a sample from the subject and determining that the level of the PLVAP protein in the sample is increased relative to a control. According to the invention, an increased level of the PLVAP protein in the sample relative to the control is indicative of the presence of HCC in the subject. An antibody that specifically binds PLVAP is used to detect the level of a PLVAP protein in a sample from the subject.

In yet another embodiment, the invention relates to an *in vivo* method of detecting HCC in a

subject (*e.g.*, a human), comprising administering a radioisotope-labeled antibody that specifically binds PLVAP by intra-arterial injection or intravenous injection, obtaining an image of the liver of the subject and detecting accumulation of the antibody in the liver of the subject. According to the invention, detection of accumulation of the antibody in the liver is indicative of HCC in the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow chart diagram depicting an algorithm for the identification of genes that show extreme differential expression between tumor and adjacent non-tumorous tissues.

FIG. 2 is a graph depicting PLVAP gene expression intensities in paired HCC (PHCC) and adjacent non-tumorous liver tissue (PN) samples (n=18), as well as unpaired HCC samples (n= 82) as determined by mRNA transcript profiling using Affymetrix gene chips.

FIG. 3A is a graph depicting relative PLVAP expression quantities in paired HCC (PHCC) and adjacent non-tumorous liver tissue (PN) samples as determined by Taqman quantitative RT-PCR. PLVAP mRNA levels are significantly higher in HCC relative to non-tumorous liver tissues.

FIG. 3B is a graph depicting PLVAP gene expression intensities in 18 paired HCC (PHCC) and adjacent non-tumorous liver tissue (PN) samples as determined by microarray analysis. PLVAP transcript levels were higher in HCC than in adjacent non-tumorous liver tissue from each individual for all individuals tested except one.

FIGS. 4A and 4B show the nucleotide sequence (SEQ ID NO:1) and the deduced amino acid sequence (SEQ ID NO:2) of the His-tagged human $PLVAP_{51-442}$ protein recombinant fusion protein used to generate mouse anti-PLVAP polyclonal antisera.

FIG. 5 is an image of a Western blot depicting the detection of recombinant PLVAP protein before and after thrombin digestion to remove the His tag. Arrows to the left of the blot indicate the locations of His-PLVAP and PLVAP on the blot. The numbers to the left of the blot indicate the positions of molecular weight standards.

FIG. 6A is a graph depicting the presence of significant relative quantities of PLVAP mRNA in HCC endothelial cells obtained by laser-capturing microdissection from two HCC tissue samples (Sample A (black) and Sample B (gray)) as determined by two-step real-time quantitative RT-PCR. Dashed lines represent Taqman quantitative RT-PCR signals from beta-actin mRNA in the same samples used for quantitative RT-PCR of PLVAP mRNA. The results indicate presence of readily measurable PLVAP mRNA in the dissected endothelial cells (solid lines).

FIG. 6B is a graph depicting the absence of significant relative quantities of PLVAP mRNA in cells obtained by laser-capturing microdissection from non-tumorous liver tissue adjacent to HCC tissue in two HCC samples (Sample A (black) and Sample B (gray)) as determined by two-step Taqman real-time quantitative RT-PCR. The results indicate no detectible (solid black line) and barely detectible (solid gray line) PLVAP mRNA in the dissected cells.

FIG. 6C is a graph depicting the relative quantities of PLVAP mRNA in HCC tumor cells obtained by laser-capturing microdissection from two HCC tissue samples (Sample A (black) and Sample B (gray)) as determined by two-step Taqman real-time quantitative RT-PCR. The results indicate presence of very small amounts of PLVAP mRNA (solid lines) in the dissected HCC cells due to unavoidable minor contamination from portion of vascular endothelial cells attached to the dissected HCC cells.

FIG. 7 is a graph depicting anti-PLVAP antibody titer in mouse antiserum raised against recombinant $PLVAP_{51-442}$ protein as determined by ELISA.

FIGS. 8A-8F are images showing sections of formalin-fixed paired HCC (FIGS. 8A, 8C, 8E) and adjacent non-tumorous liver tissues (FIGS. 8B, 8D, 8F) from three patients with hepatocellular carcinoma that were stained immunohistochemically using anti-PLVAP polyclonal antisera to detect localization of PLVAP protein. Paired tissues are shown in FIGS. 8A, 8B; FIGS. 8C, 8D; and FIGS. 8E, 8F. PLVAP protein, which appears as a brown stain (arrows) in the HCC images, was detected only in capillary endothelial cells of hepatocellular carcinomas (FIGS. 8A, 8C, 8E). No detectable HCC was present in non-tumorous liver tissue (FIGS. 8B, 8D, 8F).

FIGS. 9A-9F are images showing sections of formalin-fixed HCC (FIGS. 9A, 9C, 9E, 9F) and non-tumorous liver tissues (FIGS. 9B, 9D) from three additional patients with hepatocellular carcinoma that were stained immunohistochemically using anti-PLVAP polyclonal antisera to detect localization of PLVAP protein. FIGS. 9A, 9B and FIGS. 9C, 9D show paired tissue samples of HCC and adjacent non-tumorous liver tissue. PLVAP protein, which appears as a brown stain (arrows) in the HCC images, was detected only in capillary endothelial cells of hepatocellular carcinomas (FIGS. 9A, 9C, 9E, 9F). No detectable HCC was present in non-tumorous liver tissue (FIGS. 9B, 9D).

FIGS. 10A-10F are images showing sections of formalin-fixed focal nodular hyperplasia tissues from six different patients that were stained immunohistochemically using anti-PLVAP polyclonal antisera to detect localization of PLVAP protein. PLVAP protein was not detected in endothelial cells lining the vascular sinusoids/capillary of non-tumorous liver tissues of focal nodular hyperplasia. Some positive staining (brown) was noted in epithelial cells of bile ducts (Figs. 10A, 10D and 10F) and vessels of portal tracts (Figs. 10D and 10F), but not in the endothelial cells of liver parenchyma. The positive staining of bile duct epithelial cells was due to binding of non-specific antibodies in the PLVAP antiserum.

FIGS. 11A and 11B are images showing sections of formalin-fixed tissue from two patients with hepatic hemangioma that were stained immunohistochemically with anti-PLVAP polyclonal antiserum. Endothelial lining cells of hepatic hemangioma did not show significant expression of PLVAP protein.

FIGS. 12A and 12B are images showing sections of formalin-fixed tissue from two patients with chronic active hepatitis B that were stained immunohistochemically with anti-PLVAP polyclonal antiserum. PLVAP protein was not detected in endothelial cells lining the vascular sinusoids/capillary of non-tumorous liver tissues from chronic hepatitis B patients.

FIGS. 13A-13D are images showing sections of formalin-fixed tissue from three different patients with chronic active hepatitis C that were stained immunohistochemically with anti-PLVAP polyclonal antiserum. The tissue sections shown in FIGS. 13B and 13D are from the same patient. PLVAP protein was not detected in endothelial cells lining the vascular sinusoids/capillary of non-tumorous liver tissues from chronic hepatitis C patients.

FIGS. 14A-14D are images showing sections of formalin-fixed tissue from three different patients with metastatic liver cancers that were stained immunohistochemically with anti-PLVAP polyclonal antiserum. The tissue sections are from patients with metastatic colorectal adenocarcinoma (FIG. 14A), intrahepatic cholangiocarcinoma (FIGS. 14B and 14C) or metastatic ovarian carcinoma (FIG. 14D). The tissue sections shown in FIGS. 14B and 14C are from the same patient. PLVAP protein was not detected in endothelial cells lining the vascular sinusoids/capillary of metastatic cancer tissues.

FIG. 15A shows the nucleotide gene (top) (SEQ ID NO:3) and deduced amino acid (middle) (SEQ ID NO:4) sequences of the V_H domain of monoclonal antibody KFCC-GY4. The sequence of amino acid residues in CDRs 1 (SEQ ID NO:5), 2 (SEQ ID NO:6) and 3 (SEQ ID NO:7) also are indicated (bottom).

FIG. 15B shows the nucleotide gene (top) (SEQ ID NO:8) and deduced amino acid (middle) (SEQ ID NO:9) sequences of the V_L domain of monoclonal antibody KFCC-GY4. The sequence of amino acid residues in CDRs 1 (SEQ ID NO:10), 2 (SEQ ID NO:11) and 3 (SEQ ID NO:12) also are indicated (bottom).

FIG. 16A shows the nucleotide gene (top) (SEQ ID NO:13) and deduced amino acid (middle) (SEQ ID NO:14) sequences of the V_H domain of monoclonal antibody KFCC-GY5. The sequence of amino acid residues in CDRs 1 (SEQ ID NO:15), 2 (SEQ ID NO:16) and 3 (SEQ ID NO:17) also are indicated (bottom).

FIG. 16B shows the nucleotide gene (top) (SEQ ID NO:18) and deduced amino acid (middle) (SEQ ID NO:19) sequences of the V_L domain of monoclonal antibody KFCC-GY5. The sequence of amino acid residues in CDRs 1 (SEQ ID NO:20), 2 (SEQ ID NO:21) and 3 (SEQ ID NO:22) also are indicated (bottom).

FIG. 17 is a graph depicting the binding of KFCC-GY4 (open circles) and KFCC-GY5 (filled circles) monoclonal antibodies to recombinant PLVAP protein at various antibody concentrations, as determined by ELISA.

FIG. 18 is an immunoblot showing that KFCC-GY4 and KFCC-GY5 monoclonal antibodies can detect 5 ng of recombinant PLVAP protein. Lane 1: molecular weight standard; Lane 2: immunoblot with KFCC-GY4 monoclonal antibody; Lane 3: immunoblot with KFCC-GY5 monoclonal antibody. The molecular weight of recombinant PLVAP protein is 45kD.

FIGS. 19A and 19C are Coomassie blue-stained SDS acrylamide gels. Lane 1: molecular weight standard; Lane 2: hydrophobic membrane proteins extracted with TX-114 from human umbilical

cord vascular endothelial cells that had been stimulated with VEGF (40 ng/ml) for 72 hours before extraction.

FIG. 19B is an immunoblot, wherein the extract shown in Lane 2 of FIG. 19A was probed with KFCC-GY4 monoclonal antibodies. Lane 1: molecular weight standard; Lane 2: hydrophobic membrane proteins extracted with TX-114 from human umbilical cord vascular endothelial cells that had been stimulated with VEGF (40 ng/ml) for 72 hours before extraction.

FIG. 19D is an immunoblot, wherein the extract shown in Lane 2 of FIG. 19C was probed with KFCC-GY-5 monoclonal antibodies. Lane 1: molecular weight standard; Lane 2: hydrophobic membrane proteins extracted with TX-114 from human umbilical cord vascular endothelial cells that had been stimulated with VEGF (40 ng/ml) for 72 hours before extraction.

FIG. 20A is a fluorescence micrograph depicting immunofluorescence staining of human vascular endothelial cells (HUVEC) with control normal mouse IgG. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Magnification = 600x.

FIG. 20B is a fluorescence micrograph depicting immunofluorescence staining of human vascular endothelial cells (HUVEC) with monoclonal antibody to von Willebrand factor (VWF). VWF is a positive marker for human vascular endothelial cells. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Magnification = 600x.

FIG. 20C is a fluorescence micrograph depicting immunofluorescence staining of human vascular endothelial cells (HUVEC) with KFCC-GY4 monoclonal antibody to PLVAP. KFCC-GY4 monoclonal anti-PLVAP antibodies reacted positively with human vascular endothelial cells. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Magnification = 600x.

FIG. 20D is a fluorescence micrograph depicting immunofluorescence staining of human vascular endothelial cells (HUVEC) with KFCC-GY5 monoclonal antibody to PLVAP. KFCC-GY5 monoclonal anti-PLVAP antibodies reacted positively with human vascular endothelial cells. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Magnification = 600x.

FIG. 21A is a light micrograph of a section of formalin-fixed hepatoma tissue embedded in a paraffin block, which was stained with KFCC-GY5 monoclonal anti-PLVAP antibodies. A strong PLVAP signal (dark gray stain) was detected in vascular endothelial cells of hepatoma. Magnification is 100X.

FIG. 21B is a light micrograph of a section of formalin-fixed hepatoma tissue from the same patient as the sample shown in FIG. 21A, which was stained with KFCC-GY4 monoclonal anti-PLVAP antibodies. A moderate PLVAP signal (light gray stain) was detected in vascular endothelial cells of hepatoma. Magnification is 100X.

FIG. 21C is a light micrograph of a section of formalin-fixed hepatoma tissue from a different patient than the samples shown in FIGS. 21A and 21B, which was stained with KFCC-GY5 monoclonal anti-PLVAP antibodies. A strong PLVAP signal (dark gray stain) was detected in vascular endothelial cells. Magnification is 100X.

FIG. 21D is a light micrograph of a section of formalin-fixed hepatoma tissue from the same patient as the sample shown in FIG. 21C embedded in a paraffin block, which was stained with KFCC-GY4 monoclonal anti-PLVAP antibodies. A moderate PLVAP signal (light gray stain) was detected in vascular endothelial cells, indicating that KFCC-GY4 monoclonal antibodies bind the PLVAP antigen less well than KFCC-GY5 antibodies. Magnification is 100X.

FIGS. 22A-H are light micrographs of sections of hepatoma tissues (FIGS. 22A, 22C, 22E, and 22G) and adjacent non-tumorous liver tissues (FIGS. 22B, 22D, 22F, and 22H) from four different randomly selected hepatoma patients. The sections were stained with KFCC-GY5 monoclonal anti-PLVAP antibodies. PLVAP signal (gray stain) was detected in vascular endothelial cells of hepatoma tissue, but not in vascular endothelial cells non-tumorous liver tissue. Magnification is 100X. FIGS. 22A and 22B, 22C and 22D, 22E and 22F, and 22G and 22H represent the four sets of paired hepatoma and non-tumorous liver tissues.

FIG. 23A is a fluorescence micrograph depicting human vascular endothelial cells (HUVECs) that were stained with control mouse IgG. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

FIG. 23B is a fluorescence micrograph depicting human vascular endothelial cells (HUVECs) that were stained with KFCC-GY4 monoclonal antibody to PLVAP. KFCC-GY4 monoclonal anti-PLVAP antibodies reacted positively with the surfaces of the human vascular endothelial cells. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

FIG. 23C is a fluorescence micrograph depicting human vascular endothelial cells (HUVECs) that were stained with KFCC-GY5 monoclonal antibody to PLVAP. KFCC-GY5 monoclonal anti-PLVAP antibodies reacted positively with the surfaces of the human vascular endothelial cells. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

FIG. 24 shows the amino acid sequence of human PLVAP protein (Genbank Accession No. NP_112600; SEQ ID NO:23).

FIGS. 25A and 25B show the nucleotide sequence of full-length human PLVAP cDNA (Genbank Accession No. NM_031310; SEQ ID NO:24).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the terms "Plasmalemma Vesicle-Associated Protein," "PLVAP," and "PV-1" refer to a naturally occurring or endogenous PLVAP (*e.g.*, mammalian, human) protein, and to proteins having an amino acid sequence that is the same or substantially the same as that of naturally occurring or endogenous PLVAP protein (*e.g.*, recombinant proteins, synthetic proteins). Accordingly, the terms "Plasmalemma Vesicle-Associated Protein," "PLVAP," and "PV-1", which are used interchangeably herein, include polymorphic or allelic variants and other isoforms of a PLVAP protein produced by, *e*.g., alternative splicing or other cellular processes, that occur naturally in mammals (*e.g.,* humans). Preferably, the PLVAP protein is a human protein that has the amino acid sequence of SEQ ID NO:23 (See, Genbank Accession No. NP_112600 and FIG. 24).

As defined herein, a "PLVAP antagonist" is an agent (*e.g.*, antibody, small molecule, peptide, peptidomimetic, nucleic acid) that, in one embodiment, inhibits (*e.g.*, reduces, prevents) an activity of a PLVAP protein; or, in another embodiment, inhibits (*e.g.*, reduces, prevents) the expression of a PLVAP gene and/or gene product. Activities of a PLVAP protein that can be inhibited by an antagonist of the invention include, but are not limited to, formation, growth, vascularization and/or progression of a hepatocellular carcinoma tumor. In a particular, embodiment, the PLVAP antagonist specifically binds a mammalian (*e.g.*, human) PLVAP protein and inhibits an activity of the PLVAP protein.

As used herein, "specifically binds" refers to binding of an agent (*e.g.*, an antibody) to a PLVAP gene product (*e.g.,* RNA, protein) with an affinity *(e.g.,* a binding affinity) that is at least about 5 fold, preferably at least about 10 fold, greater than the affinity with which the PLVAP antagonist binds a non-PLVAP protein.

As used herein, the term "polypeptide" refers to a polymer of amino acids, and not to a specific length. Thus, "polypeptide" encompasses proteins, peptides, and oligopeptides.

As used herein, the term "antibody" refers to a polypeptide having affinity for a target, antigen, or epitope, and includes both naturally-occurring and engineered antibodies. The term "antibody" encompasses polyclonal, monoclonal, human, chimeric, humanized, primatized, veneered, and single chain antibodies, as well as fragments of antibodies (*e.g.*, Fv, Fc, Fd, Fab, Fab', F(ab'), scFv, scFab, dAb). (See *e.g.,* Harlow et al., Antibodies A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

The term "antibody variable region" refers to the region of an antibody that specifically binds an epitope (*e.g.*, V_H, V_{HH}, V_L), either independently or when combined with other antibody variable regions (*e.g.*, a V_H/V_L pair).

The term "epitope" refers to a unit of structure conventionally bound by an antibody V_H/V_L pair. An epitope defines the minimum binding site for an antibody and, thus, represents the target of specificity of an antibody.

The term "complementarity determining region" or "CDR" refers to a hypervariable region of an antibody variable region from a heavy chain or light chain, which contains amino acid sequences capable of specifically binding to an antigenic target (*e.g.*, epitope). A typical heavy or light chain will have three CDRs (CDR1, CDR2, CDR3), which account for the specificity of the antibody for a particular epitope.

As defined herein, the term "antigen binding fragment" refers to a portion of an antibody that contains one or more CDRs and has affinity for an antigenic determinant by itself. Non-limiting examples include Fab fragments, $F(ab)'_2$ fragments, heavy-light chain dimers, and single chain structures, such as a complete light chain or a complete heavy chain.

As used herein, the term "specificity" refers to the ability of an antibody to bind preferentially to an epitope, and does not necessarily imply high affinity.

The term "affinity" refers to a measure of the binding strength between an antibody and an antigenic determinant. Affinity depends on a number of factors, including the closeness of stereochemical fit between the antibody and antigenic determinant, the size of the area of contact between them, and the distribution of charged and hydrophobic groups.

As used herein, the term "affinity constant" or "K_d" refers to a dissociation constant used to measure the affinity of an antibody for an antigen. The lower the affinity constant, the higher the affinity of the immunoglobulin for the antigen or antigenic determinant and vice versa. Such a constant is readily calculated from the rate constants for the association-dissociation reactions as measured by standard kinetic methodology for antibody reactions.

As referred to herein, the term "competes" means that the binding of a first polypeptide (*e.g.*, antibody) to a target antigen is inhibited by the binding of a second polypeptide (*e.g.*, antibody). For example, binding may be inhibited sterically, for example by physical blocking of a binding domain or by

alteration of the structure or environment of a binding domain such that its affinity or avidity for a target is reduced.

As used herein, the term "peptide" refers to a compound consisting of from about 2 to about 100 amino acid residues wherein the amino group of one amino acid is linked to the carboxyl group of another amino acid by a peptide bond. Such peptides are typically less than about 100 amino acid residues in length and preferably are about 10, about 20, about 30, about 40 or about 50 residues.

As used herein, the term "peptidomimetic" refers to molecules which are not peptides or proteins, but which mimic aspects of their structures. Peptidomimetic antagonists can be prepared by conventional chemical methods (see *e.g.*, Damewood J.R. "Peptide Mimetic Design with the Aid of Computational Chemistry" in Reviews in Computational Biology, 2007, Vol. 9, pp. 1-80, John Wiley and Sons, Inc., New York, 1996; Kazmierski W.K., "Methods of Molecular Medicine: Peptidomimetic Protocols," Humana Press, New Jersey, 1999).

The terms "hepatocellular carcinoma," "HCC," and "hepatoma" are used interchangeably herein to refer to cancer that arises from hepatocytes, the major cell type of the liver.

As defined herein, "therapy" is the administration of a particular therapeutic or prophylactic agent to a subject (*e.g.*, a mammal, a human), which results in a desired therapeutic or prophylactic benefit to the subject.

As defined herein, a "therapeutically effective amount" is an amount sufficient to achieve the desired therapeutic or prophylactic effect under the conditions of administration, such as an amount sufficient to inhibit (*i.e.*, reduce, prevent) tumor formation, tumor growth (proliferation, size), tumor vascularization and/or tumor progression (invasion, metastasis) in the liver of a patient with HCC. The effectiveness of a therapy (*e.g.*, the reduction/elimination of a tumor and/or prevention of tumor growth) can be determined by any suitable method (*e.g.*, *in situ* immunohistochemistry, imaging (ultrasound, CT scan, MRI, NMR), ^{3}H-thymidine incorporation)

As defined herein a "treatment regimen" is a regimen in which one or more therapeutic or prophylactic agents are administered to a mammalian subject at a particular dose (*e.g.*, level, amount, quantity) and on a particular schedule or at particular intervals (*e.g.*, minutes, days, weeks, months).

As used herein, a "subject" refers to a mammalian subject. The term "mammalian subject" is defined herein to include mammals such as primates (*e.g.*, humans), cows, sheep, goats, horses, dogs cats, rabbits, guinea pigs, rats, mice or other bovine, ovine, equine, canine feline, rodent or murine species. Examples of suitable subjects include, but are not limited to, human patients who have, or are at risk for developing, HCC. Examples of high-risk groups for the development of HCC include individuals with chronic hepatitis infection (hepatitis B, hepatitis C) and individuals who have cirrhosis of the liver or related hepatic conditions.

The terms "prevent," "preventing," or "prevention," as used herein, mean reducing the probability/likelihood or risk of HCC tumor formation or progression by a subject, delaying the onset of a condition related to HCC in the subject, lessening the severity of one or more symptoms of an HCC-related condition in the subject, or any combination thereof. In general, the subject of a preventative regimen most likely will be categorized as being "at-risk", *e.g.*, the risk for the subject developing HCC is higher than the risk for an individual represented by the relevant baseline population.

As used herein, the terms "treat," "treating," or "treatment," mean to counteract a medical condition (*e.g.*, a condition related to HCC) to the extent that the medical condition is improved according to a clinically-acceptable standard (*e.g.*, reduced number and/or size of HCC tumors in a subject's liver).

As used herein, the terms "low stringency," "medium stringency," "high stringency," or "very high stringency conditions" describe conditions for nucleic acid hybridization and washing. Guidance for performing hybridization reactions can be found in Current Protocols in Molecular Biology, John Wiley &

Sons, N.Y. (1989), 6.3.1-6.3.6. Aqueous and nonaqueous methods are described in that reference and either can be used. Specific hybridization conditions referred to herein are as follows: (1) low stringency hybridization conditions in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by two washes in 0.2X SSC, 0.1% SDS at least at 50C (the temperature of the washes can be increased to 55°C for low stringency conditions); (2) medium stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60C; (3) high stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C; and preferably (4) very high stringency hybridization conditions are 0.5M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C. Very high stringency conditions (4) are the preferred conditions and the ones that should be used unless otherwise specified.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.) and chemical methods.

PLVAP

Plasmalemma vesicle-associated protein (PLVAP), also known as PV1, is a type II integral membrane glycoprotein whose expression is restricted to certain vascular endothelial cells (Mol Biol Cell 15:3615-3630 (2004)). PLVAP has been shown to be a key structural component of fenestral and stomatal diaphragms of fenestrated endothelia *Id.* In addition, PLVAP expression is necessary for the formation of endothelial fenestral diaphragms and may be involved in modulating endothelial permeability and transport (Am J Physiol Heart Circ Physiol 286:H1347-1353, 2004). The genomic organization of human PLVAP gene has been reported (Stan RV, Arden KC, Palade GE. cDNA and protein sequence, genomic organization, and analysis of cis regulatory elements of mouse and human PLVAP genes. Genomics 72;304-313, 2001).

As described herein, the inventors have demonstrated that PLVAP gene expression is significantly elevated in hepatocellular carcinoma tissues relative to adjacent non-tumorous tissues in the liver of human HCC patients. In addition, the present inventors have determined that PLVAP protein is mainly expressed in, and localizes to, vascular endothelial cells surrounding or within HCC tumors, but is not expressed in, or localized to, cells associated with other liver pathologies. Accordingly, PLVAP represents a novel target for the diagnosis and treatment of HCC.

Diagnostic and Prognostic Methods

The present invention encompasses diagnostic and prognostic methods that comprise assessing expression of PLVAP in a sample (*e.g.*, liver biopsy, fine needle aspiration sample) from a mammalian subject (*e.g.*, a mammalian subject who has a liver tumor). For diagnostic methods of the invention, expression of PLVAP in the sample, or increased expression of PLVAP in the sample relative to a suitable control, indicates that the subject has HCC, and/or that the subject is a candidate for an anti-cancer therapy using a PLVAP antagonist.

For prognostic methods of the invention, expression of PLVAP in a sample from a subject, or increased expression PLVAP in the sample relative to a suitable control, indicates a poor prognosis. The prognosis can be a prognosis for patient survival, a prognosis for risk of metastases and/or a prognosis for risk of relapse.

Suitable samples for these methods include a tissue sample, a biological fluid sample, a cell(s) (*e.g.,* a tumor cell) sample, and the like. Any means of sampling from a subject, for example, by blood

draw, spinal tap, tissue smear or scrape, or tissue biopsy can be used to obtain a sample. Thus, the sample can be a biopsy specimen (*e.g.*, tumor, polyp, mass (solid, cell)), aspirate, smear or blood sample. The sample can be a tissue from a liver that has a tumor (*e.g.*, cancerous growth) and/or tumor cells, or is suspected of having a tumor and/or tumor cells. For example, a tumor biopsy can be obtained in an open biopsy, a procedure in which an entire (excisional biopsy) or partial (incisional biopsy) mass is removed from a target area. Alternatively, a tumor sample can be obtained through a percutaneous biopsy, a procedure performed with a needle-like instrument through a small incision or puncture (with or without the aid of a imaging device) to obtain individual cells or clusters of cells (*e.g.,* a fine needle aspiration (FNA)) or a core or fragment of tissues (core biopsy). The biopsy samples can be examined cytologically (*e.g.,* smear), histologically (*e.g.,* frozen or paraffin section) or using any other suitable method (*e.g.*, molecular diagnostic methods). A tumor sample can also be obtained by *in vitro* harvest of cultured human cells derived from an individual's tissue. Tumor samples can, if desired, be stored before analysis by suitable storage means that preserve a sample's protein and/or nucleic acid in an analyzable condition, such as quick freezing, or a controlled freezing regime. If desired, freezing can be performed in the presence of a cryoprotectant, for example, dimethyl sulfoxide (DMSO), glycerol, or propanediol-sucrose. Tumor samples can be pooled, as appropriate, before or after storage for purposes of analysis. The tumor sample can be from a patient who has a liver cancer, for example, hepatocellular carcinoma.

Suitable assays that can be used to assess the presence or amount of a PLVAP in a sample (*e.g.*, biological sample) are known to those of skill in the art. Methods to detect a PLVAP protein or peptide include immunological and immunochemical methods like flow cytometry (*e.g.*, FACS analysis), enzyme-linked immunosorbent assays (ELISA), including chemiluminescence assays, radioimmunoassay, immunoblot (*e.g.*, Western blot), immunohistochemistry (IHC), and other antibody-based quantitative methods (*e.g.*, Luminex® beads-based assays). Other suitable methods include, for example, mass spectroscopy. For example, antibodies to PLVAP can be used to determine the presence and/or expression level of PLVAP in a sample directly or indirectly using, *e.g.*, immunohistochemistry (IHC). For instance, paraffin sections can be taken from a biopsy, fixed to a slide and combined with one or more antibodies by suitable methods. In a particular embodiment, detection of PLVAP protein in vascular endothelial cells surrounding hepatocytes in a sample is indicative of HCC.

Methods to detect PLVAP gene expression include PLVAP nucleic acid amplification and/or visualization. To detect PLVAP gene expression, a nucleic acid can be isolated from an individual by suitable methods which are routine in the art (see, *e.g.,* Sambrook *et al.,* 1989). Isolated nucleic acid can then be amplified (by *e.g.*, polymerase chain reaction (PCR) *(e.g.,* direct PCR, quantitative real time PCR, reverse transcriptase PCR), ligase chain reaction, self sustained sequence replication, transcriptional amplification system, Q-Beta Replicase, or the like) and visualized (by *e.g,* labeling of the nucleic acid during amplification, exposure to intercalating compounds/dyes, probes). PLVAP RNA (*e.g.*, mRNA) or expression thereof can also be detected using a nucleic acid probe, for example, a labeled nucleic acid probe (*e.g.*, fluorescence *in situ* hybridization (FISH)) directly in a paraffin section of a tissue sample taken from, *e.g.*, a tumor biopsy, or using other suitable methods. PLVAP gene expression thereof can also be assessed by Southern blot or in solution (*e.g.,* dyes, probes). Further, a gene chip, microarray, probe (*e.g.,* quantum dots) or other such device (*e.g.*, sensor, nanonsensor/detector) can be used to detect expression and/or differential expression of a PLVAP gene.

A hepatocellular carcinoma can be diagnosed by detecting expression of a PLVAP protein in a sample from a patient. Thus, the method does not require that PLVAP expression in the sample from the patient be compared to the expression of PLVAP in a control. The presence or absence of PLVAP can be ascertained by the methods described herein or other suitable assays. In another embodiment, an increase in expression of PLVAP can be determined by comparison of PLVAP expression in the sample to that of a

suitable control. Suitable controls include, for instance, a non-neoplastic tissue sample from the individual, non-cancerous cells, non-metastatic cancer cells, non-malignant (benign) cells or the like, or a suitable known or determined reference standard. The reference standard can be a typical, normal or normalized range or level of expression of a PLVAP protein (*e.g.*, an expression standard). Thus, the method does not require that expression of the protein be assessed in a suitable control.

PLVAP antibodies

As described herein, antibodies that bind PLVAP have utility in the diagnosis and treatment of HCC in human subjects. For example, antibodies that specifically bind PLVAP can be used to detect the presence of PLVAP on capillary endothelial cells of hepatocellular carcinoma in specimens of liver core biopsies or needle aspirates by immunohistochemical staining (IHC). In addition, antibodies (*e.g.*, humanized antibodies, chimeric antibodies) to PLVAP can be labeled with a proper tracer (*e.g.*, radioisotope) for immuno-positron emission tomography (immuno-PET) (Clin Cancer Res 12:1958-1960, 2006;Clin Cancer Res 12:2133-2140, 2006) to determine whether a space occupying lesion(s) in the liver of a subject is hepatocellular carcinoma. Anti-PLVAP antibodies (*e.g.*, humanized antibodies) can also be labeled with a cytotoxic agent (radioactive or nonradioactive) for therapeutic purposes (Weiner LM, Adams GP, Von Mehren M. Therapeutic monoclonal antibodies: General principles. In: Cancer: Principles & Practice of Oncology. 6th ed. DeVita VT, Hellman S, Rosenberg SA, eds. Philadelphia: Lippincott Williams & Wilkins; 2001:495-508.; Levinson W, Jawetz E. Medical Microbiology & Immunology. 4th ed. Stamford: Appleton & Lange; 1996:307-47. ; Scheinberg DA, Sgouros G, Junghans RP. Antibody-based immunotherapies for cancer. In: Cancer Chemotherapy & Biotherapy: Principles and Practice. 3rd ed. Chabner BA, Longo DL, eds. Philadelphia: Lippincott Williams & Wilkins; 2001:850-82).

Accordingly, in one embodiment, the invention provides an antibody that binds (*e.g.*, specifically binds) a PLVAP protein (*e.g.,* a human PLVAP protein (SEQ ID NO:23)). Antibodies that specifically bind to a PLVAP protein can be polyclonal, monoclonal, human, chimeric, humanized, primatized, veneered, and single chain antibodies, as well as fragments of antibodies (*e.g.*, Fv, Fc, Fd, Fab, Fab', F(ab'), scFv, scFab, dAb), among others. (See *e.g.,* Harlow et al., Antibodies A Laboratory Manual, Cold Spring Harbor Laboratory, 1988). Antibodies that specifically bind to a PLVAP protein can be produced, constructed, engineered and/or isolated by conventional methods or other suitable techniques. For example, antibodies which are specific for a PLVAP protein can be raised against an appropriate immunogen, such as a recombinant mammalian (*e.g.*, human) PLVAP protein (*e.g.,* SEQ ID NO:23) or a portion thereof *(e.g.,* SEQ ID NO:2) (including synthetic molecules, *e.g.*, synthetic peptides). A variety of such immunization methods have been described (see *e.g.,* Kohler et al., Nature, 256: 495-497 (1975) and Eur. J. Immunol. 6: 511-519 (1976); Milstein et al., Nature 266: 550-552 (1977*)*; Koprowski et al., U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); Current Protocols In Molecular Biology, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. et al., Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Antibodies can also be raised by immunizing a suitable host *(e.g.,* mouse) with cells that express PLVAP *(e.g.,* cancer cells/cell lines) or cells engineered to express PLVAP *(e.g.,* transfected cells). (See *e.g.,* Chuntharapai et al., J. Immunol., 152:1783-1789 (1994); Chuntharapai et al. U.S. Patent No. 5,440, 021).

At an appropriate time after immunization, *e.g.*, when the antibody titers are highest, antibody-producing cells can be obtained from the immunized animal and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (Nature 256:495-497, 1975), the human B cell hybridoma technique (Kozbor et al., Immunol. Today 4:72, 1983), the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985) or trioma techniques. The technology for producing hybridomas is well known (see

generally Current Protocols in Immunology, Coligan et al., (eds.) John Wiley & Sons, Inc., New York, NY, 1994). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds a polypeptide described herein.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating a monoclonal antibody to a polypeptide of the invention (see, *e.g.*, Current Protocols in Immunology, supra; Galfre et al., Nature, 266:55052, 1977; R.H. Kenneth, in Monoclonal Antibodies: A New Dimension In Biological Analyses, Plenum Publishing Corp., New York, New York, 1980; and Lerner, Yale J. Biol. Med. 54:387-402, 1981). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods that also would be useful.

In one alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal antibody to a PLVAP protein can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with the target polypeptide to thereby isolate immunoglobulin library members that bind the polypeptide. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia Recombinant Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene SurfZAP™ Phage Display Kit, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al., Bio/Technology 9:1370-1372, 1991; Hay et al., Hum. Antibodies Hybridomas 3:81-85, 1992; Huse et al., Science 246:1275-1281,1989; and Griffiths et al., EMBO J. 12:725-734, 1993.

Antibody fragments (*e.g.*, antigen-binding fragments) can be produced by enzymatic cleavage or by recombinant techniques. For example, papain or pepsin cleavage can generate Fab or $F(ab')_2$ fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or $F(ab')_2$ fragments.

Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons has been introduced upstream of the natural stop site. For example, a chimeric gene encoding a $F(ab')_2$ heavy chain portion can be designed to include DNA sequences encoding the CH_1 domain and hinge region of the heavy chain.

Single chain, human, chimeric, humanized, primatized (CDR-grafted), or veneered antibodies comprising portions derived from different species, are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, *e.g.,* Cabilly et al. , U.S. Patent No. 4,816,567; Cabilly *et al.,* European Patent No. 0,125,023 B1; Boss et al. , U.S. Patent No. 4,816,397; Boss *et al.,* European Patent No. 0,120,694 B1; Neuberger, M.S. et al., WO 86/01533; Neuberger, M.S. *et al.,* European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.,* European Patent No. 0 451 216 B1; and Padlan, E.A. et al. , EP 0 519 596 A1. See also, Newman, R. et al., BioTechnology, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner et al., U.S. Patent No. 4,946,778 and Bird, R.E. et al. , Science, 242: 423-426 (1988)) regarding single chain antibodies.

In a particular embodiment, the invention relates to chimeric antibodies that specifically bind to PLVAP (*e.g.*, a human PLVAP protein comprising SEQ ID NO:23). In one embodiment, chimeric antibody of

the invention comprises at least one heavy chain and at least one light chain (*e.g.*, kappa light chain) of human IgG4.

In another embodiment, the invention relates to humanized antibodies that specifically bind to PLVAP (*e.g.*, a human PLVAP protein comprising SEQ ID NO:23). Humanized antibodies can be produced using synthetic or recombinant DNA technology using standard methods or other suitable techniques. Nucleic acid (*e.g.,* cDNA) sequences coding for humanized variable regions can also be constructed using PCR mutagenesis methods to alter DNA sequences encoding a human or humanized chain, such as a DNA template from a previously humanized variable region (see *e.g.,* Kamman, M., et al. , Nucl. Acids Res., 17: 5404 (1989)); Sato, K., et al. , Cancer Research, 53: 851-856 (1993); Daugherty, B.L. et al. , Nucleic Acids Res., 19(9): 2471-2476 (1991); and Lewis, A.P. and J.S. Crowe, Gene, 101: 297-302 (1991)). Using these or other suitable methods, variants can also be readily produced. In one embodiment, cloned variable regions (*e.g.*, dAbs) can be mutated, and sequences encoding variants with the desired specificity can be selected (*e.g.*, from a phage library; see *e.g.,* Krebber et al. , U.S. 5,514,548; Hoogenboom et al., WO 93/06213, published April 1, 1993). Humanized antibodies can also be produced by and/or obtained from commercial sources including, for example, Antitope Limited (Cambridge, UK).

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select a recombinant antibody or antibody-binding fragment (*e.g.,* dAbs) from a library (*e.g.,* a phage display library), or which rely upon immunization of transgenic animals (*e.g.,* mice). Transgenic animals capable of producing a repertoire of human antibodies are well-known in the art (*e.g.*, Xenomouse® (Abgenix, Fremont, CA)) and can be produced using suitable methods (see *e.g.*, Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90: 2551-2555 (1993); Jakobovits et al., Nature, 362: 255-258 (1993); Lonberg et al., U.S. Patent No. 5,545,806; Surani et al. , U.S. Patent No. 5,545,807; Lonberg et al. , WO 97/13852).

Once produced, an antibody specific for PLVAP can be readily identified using methods for screening and isolating specific antibodies that are well known in the art. See, for example, Paul (ed.), Fundamental Immunology, Raven Press, 1993; Getzoff et al., Adv. in Immunol. 43:1-98, 1988; Goding (ed.), Monoclonal Antibodies: Principles and Practice, Academic Press Ltd., 1996; Benjamin et al., Ann. Rev. Immunol. 2:67-101, 1984. A variety of assays can be utilized to detect antibodies that specifically bind to PLVAP proteins. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assays, inhibition or competition assays, and sandwich assays.

The antibodies of the invention have a high binding affinity for PLVAP. Such antibodies will have an affinity (*e.g.*, binding affinity) for PLVAP, expressed as K_d, of at least about 10^{-7} M (*e.g.*, about 0.4 X 10^{-7} M, about 0.6 X 10^{-7} M, or higher, for example, at least about 10^{-8} M, at least about 10^{-9} M, or at least about 10^{-10} M. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, G., Ann. NY Acad. Sci. 51: 660-672, 1949). Binding affinity can also be determined using a commercially available biosensor instrument (BIACORE, Pharmacia Biosensor, Piscataway, N.J.), wherein protein is immobilized onto the surface of a receptor chip. See, Karlsson, J. Immunol. Methods 145:229-240, 1991 and Cunningham and Wells, J. Mol. Biol. 234:554-563, 1993. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding.

The antibodies of the present invention can include a label, such as, for example, a detectable label that permits detection of the antibody, and proteins bound by the antibody (*e.g.*, PLVAP), in a biological sample. A detectable label is particularly suitable for diagnostic applications. For example, a PLVAP antibody

can be labeled with a radioactive isotope (radioisotope), which can be detected by one of skill in the art using a gamma counter, a scintillation counter or by autoradiography or other suitable means. Isotopes which are useful for the purpose of the present invention include, but are not limited to: ^{3}H, ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe and ^{75}Se.

Antibodies of the invention can also be labeled with a fluorescent compound (e.g., dyes). When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to the fluorescence of the compound. Among the most commonly used fluorescent labels are fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. the antibodies of the invention can also be labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody molecule using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA), tetraaza-cyclododecane-tetraacetic acid (DOTA) or ethylenediaminetetraacetic acid (EDTA).

The antibodies of the present invention also can be coupled to a chemiluminescent compound. Examples of useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Useful bioluminescent compounds for purposes of labeling antibodies are luciferin, luciferase and aequorin.

Detection of the labeled antibodies can be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods which employ a substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of the enzymatic reaction of a substrate to similarly prepared standards.

Accordingly, the antibodies of the present invention can also be used as a stain for tissue sections. For example, a labeled antibody that binds to PLVAP can be contacted with a tissue sample, *e.g.*, a liver tissue biopsy or fine needle aspirate from a patient. This section may then be washed and the label detected using an appropriate means.

For the purpose of treating HCC, PLVAP antibodies of the invention may include a radiolabel or other therapeutic agent that enhances destruction of cells expressing PLVAP (*e.g.*, vascular endothelial cells surrounding HCC cells). Examples of suitable radioisotope labels for use in HCC therapy include, but are not limited to, ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{217}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{111}In and ^{118}Re. Optionally, a label that emits a and β particles upon bombardment with neutron radiation, such as boron, can be used as a label for therapeutic PLVAP antibodies.

Therapeutic antibodies also may include a cytotoxic agent that is capable of selectively killing cells that express PLVAP. For example, bacterial toxins such as diphtheria toxin, or ricin can be used. Methods for producing antibodies comprising fragment A of diphtheria toxin are taught in U.S. Pat. No. 4,675,382 (1987). Diphtheria toxin contains two polypeptide chains. The B chain binds the toxin to a receptor on a cell surface. The A chain actually enters the cytoplasm and inhibits protein synthesis by inactivating elongation factor 2, the factor that translocates ribosomes along mRNA concomitant with hydrolysis of ETP. See Darnell, J. et al., in Molecular Cell Biology, Scientific American Books, Inc., page 662 (1986). Alternatively, an antibody comprising ricin, a toxic lectin, may be prepared. Other suitable cytotoxic agents are know by those of skill in the art.

For *in vivo* detection, PLVAP antibodies of the invention may be conjugated to radionuclides either directly or by using an intermediary functional group. An intermediary group which is often used to bind

radioisotopes, which exist as metallic cations, to antibodies is diethylenetriaminepentaacetic acid (DTPA) or tetraazacyclododecane-tetraacetic acid (DOTA). Typical examples of metallic cations which are bound in this manner are ^{99}Tc ^{123}I, ^{111}In, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga, and ^{68}Ga.

Moreover, the antibodies of the invention may be tagged with an NMR imaging agent which include paramagnetic atoms. The use of an NMR imaging agent allows the *in vivo* diagnosis of the presence of and the extent of HCC in a patient using NMR techniques. Elements which are particularly useful in this manner are ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

Diagnostic Kits

The invention also provides diagnostic kits for detecting the presence of a hepatocellular carcinoma in a subject.

The kits of the invention include an antibody that specifically binds a PLVAP protein (*e.g.*, a human PLVAP protein). Such antibodies include any of the PLVAP antibodies of the invention described herein. In one embodiment, the antibody comprises a V_H domain having the amino acid sequence of SEQ ID NO:4 and a V_L domain having the amino acid sequence of SEQ ID NO:9. In another embodiment, the antibody comprises a V_H domain having the amino acid sequence of SEQ ID NO:14 and a V_L domain having the amino acid sequence of SEQ ID NO:19.

The diagnostic agents in the kits of the invention can include one or more labels (*e.g.*, detectable labels). Numerous suitable labels for diagnostic agents are known in the art and include, but are not limited to, any of the labels described herein. In a particular embodiment, the diagnostic agent (*e.g.*, antibody) includes a radioisotope, such that agent can be used for immuno-positron emission tomography (immuno-PET).

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

Exemplification

Example 1: PLVAP expression is elevated in HCC liver tissues relative to non-HCC liver tissues

Materials and Methods:

Tissue samples

Tissues of HCC and adjacent non-tumorous liver were collected from fresh specimens surgically removed from human patients for therapeutic purpose. These specimens were collected under direct supervision of attending pathologists. The collected tissues were immediately stored in liquid nitrogen at the Tumor Bank of the Koo Foundation Sun Yat-Sen Cancer Center (KF-SYSCC). Paired tissue samples from eighteen HCC patients were available for the study. The study was approved by the Institutional Review Board and written informed consent was obtained from all patients. The clinical characteristics of the eighteen HCC patients from this study are summarized in Table 1.

Table 1: Clinical data for eighteen HCC patients from which paired HCC and adjacent non-tumorous liver tissue samples were obtained

Case No	Sex	Age	HBsAg	HBsAb	HCV IgG	TNM Stage	AFP (ng/ml)	Differentiation
1	M	70	+	-	-	2	2	Moderate

Case No	Sex	Age	HBsAg	HBsAb	HCV IgG	TNM Stage	AFP (ng/ml)	Differentiation
2	M	75	-	+	+	4A	5	Well
3	M	59	+	-	-	4A	1232	Moderate
4	F	53	+	-	+	1	261	Moderate
5	M	45	+	-	-	2	103	Moderate
6	M	57	+	+	-	2	5	Moderate
7	M	53	+	+	-	3A	19647	Moderate
8	M	54	-	-	+	3A	7	Moderate
9	M	44	+	-	-	4A	306	Moderate
10	M	76	-	-	+	3A	371	Moderate
11	F	62	+	-	-	3A	302	Moderate
12	F	73	-	-	+	2	42	Moderate
13	M	46	+	-	-	4A	563	Moderate
14	M	45	-	-	-	3A	64435	Moderate
15	M	41	+	-	-	2	33.9	Well
16	M	44	+	+	-	2	350	Moderate
17	M	67	+	-	-	3A	51073	Moderate
18	M	34	+	-	-	4A	2331	Moderate

mRNA transcript profiling

Total RNA was isolated from tissues frozen in liquid nitrogen using Trizol reagents (Invitrogen, Carlsbad, CA). The isolated RNA was further purified using RNAEasy Mini kit (Qiagen, Valencia, CA), and its quality assessed using the RNA 6000 Nano assay in an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). All RNA samples used for the study had an RNA Integrity Number (RIN) greater than 5.7 (8.2 ± 1.0, mean ± SD). Hybridization targets were prepared from 8 µg total RNA according to Affymetrix protocols and hybridized to an Affymetrix U133A GeneChip, which contains 22,238 probe-sets for approximately 13,000 human genes. Immediately following hybridization, the hybridized array underwent automated washing and staining using an Affymetrix GeneChip fluidics station 400 and the EukGE WS2v4 protocol. Thereafter, U133A GeneChips were scanned in an Affymetrix GeneArray scanner 2500.

Determination of Present and Absent Call of Microarray Data

Affymetrix Microarray Analysis Suite (MAS) 5.0 software was used to generate present calls for the microarray data for all 18 pairs of HCC and adjacent non-tumor liver tissues. All parameters for present call determination were default values. Each probe-set was determined as "present", "absent" or "marginal" by MAS 5.0. Similarly, the same microarray data were processed using dChip version-2004 software to determine "present", "absent" or "marginal" status for each probe-set on the microarrays.

Identification of Probe-sets with Extreme Differential Expression

For identification of genes with extreme differential expression between HCC and adjacent non-tumor liver tissue, software written using Practical Extraction and Report Language (PERL) was used according to the following rules: "Tumor-specific genes" were defined as probe-sets that were called "present" in HCC and "absent" or "marginal" in the adjacent non-tumor liver tissue by both MAS 5.0 and dChip. "Non-tumor liver tissue-specific genes" were defined as probe-sets called 'absent' or 'marginal' in HCC and 'present' in the paired adjacent non-tumor liver tissue by both MAS 5.0 and dChip. A flowchart diagram depicting the identification algorithm is shown in FIG. 1.

Real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

TaqMan™ real-time quantitative reverse transcriptase-PCR(qRT-PCR) was used to quantify mRNA. cDNA was synthesized from 8 µg of total RNA for each sample using 1500 ng oligo(dT) primer and 600 units SuperScript™ II Reverse Transcriptase from Invitrogen (Carlsbad, CA) in a final volume of 60 µl according to the manufacturer's instructions. For each RT-PCR reaction, 0.5 µl cDNA was used as template in a final volume of 25 µl following the manufacturers' instructions (ABI and Roche). The PCR reactions were carried out using an Applied Biosystems 7900HT Real-Time PCR system. Probes and reagents required for the experiments were obtained from Applied Biosystems (ABI) (Foster City, CA). The sequences of primers and the probes used for real-time quantitative RT-PCR of PLVAP are 5'-CCTGCAGGCATCCCTGTA-3' (forward primer) (SEQ ID NO:25); 5'-CGGGCCATCCCTTGGT-3' (reverse primer) (SEQ ID NO:26); and 5'-CCCCATCCAGTGGCTG-3' (probe) (SEQ ID NO:27). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping gene was used as an endogenous reference for normalization. All samples were run in duplicate on the same PCR plate for the same target mRNA and the endogenous reference HPRT mRNA. The relative quantities of target mRNAs were calculated by comparative Ct method according to manufacturer's instructions (User Bulletin #2, ABI Prism 7700 Sequence Detection System). A non-tumorous liver sample was chosen as the relative calibrator for calculation.

Results:

The PLVAP gene expression intensities in 18 pairs of HCC and adjacent non-tumorous liver tissues are shown in FIG. 2. The average gene expression intensities were 759.8±436.5 and 170.6±53.4 (mean ±SD) for paired HCC and adjacent non-tumorous liver tissue, respectively. The p value of paired t-test between the two groups was 2.8×10^{-5}. These results indicate that PLVAP is expressed in HCC and not in non-tumorous liver tissue. This elevated expression of PLVAP in HCC was further confirmed when 82 unpaired HCC samples showed an average expression intensity of 810.4±482.0 (mean±SD), which is essentially the same as the finding from the 18 paired HCC samples (p=0.62 by t-test) (FIG. 2).

In order to confirm that PLVAP is significantly expressed in HCC liver tissue and not in non-tumorous liver tissue, real-time quantitative RT-PCR was performed on RNA samples from 18 pairs of HCC and adjacent non-tumorous liver tissue. Quantities of PLVAP mRNA were significantly higher in HCC relative to non-tumorous liver tissues (see FIG. 3A and Table 2). Although the results showed some overlap between two groups, PLVAP transcripts were higher in HCC than in adjacent non-tumorous liver tissue within the same individual for all individuals tested except one (FIG. 3B). This exception was likely associated with uneven degrees of RNA degradation during storage process of tissues.

Table 2: PLVAP gene expression intensities for 18 pairs of HCC and adjacent non-tumorous liver tissue.

	Expression Intensity*	
Sample Number	**HCC**	**Adjacent non-tumorous liver tissue**

	Expression Intensity*	
Sample Number	**HCC**	**Adjacent non-tumorous liver tissue**
1	1757	195
2	1329	210
3	1148	168
4	1130	211
5	1096	213
6	1068	181
7	932	101
8	804	60
9	630	155
10	612	175
11	607	125
12	519	146
13	478	300
14	422	180
15	275	105
16	251	204
17	251	155
18	186	184

Example 2: PLVAP is specifically expressed by HCC vascular endothelial cells

Materials and Methods:

Laser Capture Microdissection (LCM) of formalin-fixed paraffin embedded tissues

LCM of formalin fixed tissue from paraffin blocks was carried out using Arcturus PixCell^{R} IIe system, CapSure™ HS LCM caps, and Paradise™ reagent system from Arcturus Bioscience, Inc. (Mountain View, CA). Seven micrometer thick tissue sections were cut, deparaffinized, rehydrated, stained and dehydrated for LCM according to manufacturer's instructions. Target cells were captured onto CapSure™ HS LCM caps using 7.5 µm laser spot size at 50 mW power and 1.3 ms duration. Approximately, 5000 to 6000 cells were captured on each cap. However, only 1000 to 2000 hepatocellular carcinoma vascular endothelial cells were captured onto each cap due to paucity of cells.

RNA Extraction from LCM Tissue Sections for quantitative RT-PCR

Cells captured onto the CapSure™ HS LCM caps as described above were processed for RNA extraction, cDNA synthesis, *in vitro* transcription and antisense RNA amplification using the Paradise™

reagent system according to manufacturer's instructions. The synthesized anti-sense RNA was then used as a template for two-step TaqMan real time quantitative RT-PCR for quantitation of PLVAP and beta-actin mRNA in the cells captured by LCM. The first step (*i.e.*, reverse transcription) was carried out using 4.5 µl anti-sense RNA and TaqMan Reverse Transcription Reagents (ABI) in a final volume of 10 µl following the manufacturer's protocol. The second step (*i.e.*, real-time PCR) was performed using 2.4 µl of cDNA template, the primers/probe mix and the TaqMan universal PCR Master Mix from Applied Biosystems in a final volume of 25 µl. Real-time PCR was carried out in a Smart Cycler II (Cephid, Inc., Sunnyvale, CA). The reactions were initially incubated at 50°C for 2 minutes and then at 95°C for 10 minutes. Thereafter, 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 40 seconds were performed. The sequences of the primers and the probes are listed in Table 3.

Table 3. Primer and probe sequences for real-time quantitative RT-PCR for PLVAP and beta-actin levels in samples prepared by laser-captured microdissection.

	PLVAP gene	beta-Actin gene
forward primer	5'-CCTTGAGCGTGAGTGTTTCCA-3' (SEQ ID NO:28)	5'-GTCCCCCAACTTGAGATGTATGAA G-3' (SEQ ID NO:29)
reverse primer	5'-GGCAGGGCTGGGAGTTG-3' (SEQ ID NO:30)	5'-GTCTCAAGTCAGTGTACAGGTAAG C-3' (SEQ ID NO:31)
Taqman probe	5'-CTCCCAGGGAGACCAA-3' (SEQ ID NO:32)	5'-AAGGAGTGGCTCCCCTCC-3' (SEQ ID NO:33)

Preparation of Expression Vector for Recombinant Fusion PLVAP$_{51-442}$ Protein

Plasmid pGEM®-T Easy -PLVAP$_{51-442}$ was generated by inserting a PCR fragment encoding amino acid residues 51 to 442 of PLVAP into the pGEM®-T Easy Vector (Promega, Inc., Madison, WI). The PCR fragment was amplified from a cDNA clone of PLVAP from OriGene (Rockville, MD) by using the primer set of 5'-CATATGAACGTGCACGTGAGCACAGAGTCC-3' (SEQ ID NO:34) and 5'-GGATCCTGAGCATATCCCTGCATCCTCC-3' (SEQ ID NO:35). For construction of plasmid pET-15b-PLVAP$_{51-442}$, a cDNA fragment encoding amino acid residues 51 to 442 of PLVAP with NdeI and BamHI recognition sequences at each respective end was excised from pGEM®-T Easy -PLVAP$_{51-442}$ and inserted into pET-15b (Novagen, Inc., San Diego, CA). The expression construct described above was verified by DNA sequencing.

Expression and purification of Recombinant Fusion PLVAP$_{51-442}$ Protein

For production of recombinant His-tagged PLVAP$_{51-442}$ protein (SEQ ID NO:2) (FIG. 4), *Escherichia coli* (Rosetta-gami2(DE3)pLysS) (Novagen) was transformed by incubating competent cells with pET-15b-PLVAP$_{51-442}$ plasmid DNA on ice for 5 min, followed by incubation in a 42°C water bath for 30s and then again on ice for 2 min. Prior to plating on selective medium, the transformants were incubated at 37°C while shaking at 250 rpm with SOC medium (0.5% Yeast Extract; 2% Tryptone; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM Glucose) for 60 min. Expression of His-tagged fusion protein in Rosettagami2(DE3)pLysS *Escherichia coli* was induced with 1 mM isopropyl-ß-D-thiogalactopyranoside for 16 hours at 30°C. Following the induction, the bacterial cells were subjected to lysis by sonication in equilibration buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7) supplemented with 8 M urea and separated into soluble and insoluble fractions by centrifugation at 5,600 x g for 30 minutes. For further purification of the His-PLVAP$_{51-442}$ protein, soluble fraction was loaded on a TALON® Metal Affinity Resin

(Clontech, Inc., Palo Alto, CA), washed with equilibration buffer and eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7,250 mM imidazole). The His-tag of the purified fusion protein was removed by thrombin cleavage (Novagen) according to manufacturer's instructions (see FIG. 5). The resulting $PLVAP_{51-442}$ protein was recovered by extensive dialysis against PBS. To verify the identity of the recombinant PLVAP protein, a small quantity of mouse antiserum against GST-$PLVAP_{331-430}$ fusion protein was purchased from the Biodesign Insitute (Tempe, AZ). The recombinant $PLVAP_{51-442}$ protein without the His-tag was detected by Western blot analysis using this antibody, but did not react with antibodies to the His-tag. These results confirm the identity of the recombinant PLVAP protein.

Generation of mouse anti-human PLVAP serum

Purified $PLVAP_{51-442}$ recombinant protein in PBS was used to immunize 6 weeks old Balb/cByj mice. Each mouse was initially immunized with subcutaneous injection at multiple sites with a total of 14 µg $PLVAP_{51-442}$ protein in complete Freund's adjuvant (Sigma, Inc., St Louis, MO). Thereafter, immunization was boosted with 7 µg $PLVAP_{51-442}$ recombinant protein in incomplete Freund's adjuvant once every two weeks for three times. A week after the last boosting immunization, mice were bled for preparation of antiserum.

Enzyme-linked immunosorbent assay (ELISA)

Reagents and Solutions:

1. Recombinant PLVAP protein

2. Anti-mouse IgG-alkaline phosphatase conjugate (Cat. #: AP124A, CHEMICON)

3. Coating buffer (0.137 M Sodium Chloride, 0.01 M Sodium Phosphate Dibasic Heptahydrate, 2 mM Potassium Phosphate Monobasic, 0.002% (0.3 mM) Sodium azide , pH 7.2-7.4)

4. Washing buffer (0.137 M Sodium Chloride, 0.01 M Sodium Phosphate Dibasic Heptahydrate, 2 mM Potassium Phosphate Monobasic, 0.2% Tween20 (Cat. P1379, SIGMA, pH 7.2-7.4)

5. Blocking buffer (0.137 M Sodium Chloride, 0.01 M Sodium Phosphate Dibasic Heptahydrate, 2 mM Potassium Phosphate Monobasic, 2% Bovine Serum Albumin (Cat. 82-045, PENTEX), 0.05% Tween20 (Cat. P1379, SIGMA), pH 7.2-7.4)

6. Carbonate buffer (0.016 M Sodium Bicarbonate, 0.014 M Sodium Carbonate 2 mM Magnesium Chloride, 0.002% (0.3 mM) Sodium Azide, pH 9.6)

7. Akaline Phosphatase substrate: One 40 mg phosphatase substrate tablet (Cat. P5994, SIGMA) dissolved in 40 ml carbonate buffer

Procedure:

The titers of antibodies in the anti-PLVAP sera were determined using ELISA. First, the 96 well ELISA plate was coated with 50 µl of PLVAP protein dissolved in Phosphate buffered saline (PBS) containing 0.002 % sodium azide (i.e., coating buffer) at a concentration in the range of 2.5 µg/ml overnight at 4°C. After three washes with 200 µl of washing buffer (PBS containing 0.05% Tween-20), each well of the coated plate was blocked with 150 µl blocking buffer (i.e., washing buffer containing 2% bovine serum albumin) at room temperature for 30 minutes. After three further washes, each well was incubated with 50 µl of diluted

antiserum (serial two fold dilution from 1,000x to 128,000x) prepared in the dilution buffer for 45 minutes at room temperature. Thereafter, each well was incubated with anti-mouse IgG alkaline phosphatase conjugate at 5,000X dilution (Chemico, Inc., Temecula, CA) for 30 minutes at room temperature. After three washes, the bound antibodies were quantified with 100 µl alkaline phosphatase substrate (Sigma, Inc., St Louis, MO) and measurement of absorbance was performed at 405 nm after an incubation period of 25 to 40 min. using an ELISA plate reader.

Immunohistochemical (IHC) detection of PLVAP in formalin-fixed tissues

Six micrometer sections were cut from paraffin blocks of formalin-fixed tissues. The sections were mounted on SuperFrost plus adhesion glass slides (Menzel Glaser GmbH, Braunschweig, Germany). The sections then were processed for immunostaining of PLVAP in a Benchmark XT automated staining instrument (Ventana Medical Systems, Inc., Tucson, AZ) using XT-iView-DAB-V.1 protocol with mild CCI conditioning for 30 minutes and sections were incubated with 400X diluted anti-human PLVAP serum at 37°C for 36 minutes. The second antibody and the reagents used to detect binding of mouse anti-human PLVAP antibodies were from the *i*View™DAB Detection Kit from Ventana Medical Systems, Inc. (Tucson, AZ). All reagents and buffers were purchased from Ventana Medical Systems.

Results:

To determine the cellular source of PLVAP in HCC samples, HCC vascular endothelial cells, tumor cells of hepatocellular carcinoma and non-tumorous hepatocytes, including lining sinusoidal endothelial cells, were dissected out of the samples using laser capture microdissection (LCM). Due to close apposition between hepatoma cells and capillary-lining endothelial cells, effort was made to avoid inclusion of capillary-lining endothelial cells during dissection. The RNAs extracted from the dissected cells were used for two-step real time quantitative RT-PCR to determine the relative quantities of PLVAP mRNA. Specimens from two different patients were studied. The results shown in Table 4 and FIGS. 6A-C indicate that PLVAP is expressed by HCC vascular endothelial cells (FIG. 6A), while no detectable PLVAP transcript was detected in adjacent non-tumorous liver tissues (FIG. 6B).

Table 4: Determination of PLVAP mRNA relative quantities in two HCC samples by Taqman real time quantitative RT-PCR in cells dissected by laser-capturing microdissection

	Relative Quantity of PLVAP mRNA		
HCC Sample	**HCC Endothelial Cells**	**Adjacent Non-tumorous Liver Tissue**	**HCC Tumor Cells**
A	1	0	0.002
B	1	0.001	0.057

In order to further investigate the tissue and disease specificity of PLVAP expression, polyclonal antibodies for use in immunohistochemistry (IHC) studies were generated against the extracellular domain of human PLVAP (amino acids 51 to 442). As shown in FIG. 7, antiserum obtained from Balb/c mice that were immunized with recombinant $PLVAP_{51-442}$ protein contained a high titer of anti-PLVAP antibodies.

The anti-PLVAP antiserum was then used to determine the localization of PLVAP expression in tissue sections from patients with hepatocellular carcinoma (n=7) (FIGS.8A-F and 9A-F), focal nodular hyperplasia (n=4) (FIGS. 10A-F), hepatic hemangioma (n=2) (FIGS. 11A and B), chronic active hepatitis B (n=2) (FIGS. 12A and B) or C (n=4) (FIGS. 13A-D), and metastatic cancer (n=4) (*i.e.*, intrahepatic cholangiocarcinoma, metastatic colorectal adenocarcinoma, or metastatic ovarian carcinoma) (FIGS. 14A-

D). The results showed that only capillary endothelial cells of hepatocellular carcinomas expressed PLVAP protein (FIGS. 8A,C,E and 9A,C,E,F). PLVAP protein was not expressed by endothelial cells lining the vascular sinusoids/capillary of non-tumorous liver tissues including cirrhotic liver, liver of focal nodular hyperplasia (FIGS. 10A-F), and chronic hepatitis (FIGS. 12A and B; FIGS. 13A-D). Endothelial lining cells of hepatic hemagioma did not show significant expression of PLVAP, either (FIGS. 11A and B). These results demonstrate that PLVAP is a vascular endothelial biomarker that is specific for hepatocellular carcinoma, but not for other diseases of liver. Therefore, PLVAP can be used as a diagnostic marker and therapeutic target for HCC.

Example 3: Production and characterization of mouse monoclonal antibodies that specifically bind PLVAP

Materials and Methods

Immunization Procedures

Five six-week-old female Balb/cByJ mice were immunized initially with 20 µg of purified recombinant PLVAP protein dissolved in 0.125 mL phosphate buffered saline (PBS) and emulsified in an equal volume of complete Freund's adjuvant. The PLVAP-adjuvant mixture was injected in 0.05 mL volumes into each of four separate subcutaneous sites on the ventral side of the mice near the axillary and inguinal lymphatics, as well as a fifth subcutaneous site, which was located between the scapulae. All mice received a booster immunization of 20 µg of recombinant PLVAP protein injected intraperitoneally three times every two weeks. One week after the last booster immunization, test bleedings were taken to measure whether mice were producing sufficiently high titers of anti-PLVAP antibodies (>10,000X). A solid-phase enzyme-linked immunosorbent assay (ELISA) was used for this purpose. The mouse that produced the highest titer of PLVAP antibody was selected for the production of hybridomas.

Development of Murine Monoclonal Anti-PLVAP Antibodies

Three days before the scheduled fusion experiment to produce hybridomas, the mouse that produced the highest titer of PLVAP antibody was injected intravenously with 20 µg of recombinant PLVAP. Hybridomas producing monoclonal antibodies (MAbs) against PLVAP were produced according to a previously described protocol (see Unit 2.5 Production of Monoclonal Antibodies, in Current Protocols in Immunology, editors: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, and Strober W. Published by John Wiley & Sons, Inc., New York, 2001) with minor modification. Specifically, spleen cells harvested from the immunized mouse were fused with SP2/0 myeloma cells at a ratio of 7.5:1 (spleen cell: myeloma cells) using 50% polyethylene glycol 1540. The fusion products were seeded into 96-well flat-bottom tissue culture plates, and hypoxanthine-aminopterinthymidine (HAT) selective medium was added the next day. Seven to ten days later, the supernatants of growth-positive wells were screened for production of anti-PLVAP antibodies by ELISA. Hybridomas initially producing anti-PLVAP MAbs were expanded and re-screened. Hybridomas that showed continued production of antibodies were cloned by the limiting dilution method. MAb isotypes were determined using an ELISA. Monoclonal antibodies were purified from ascites or culture media by Protein G affinity column chromatography (Unit 2.7 Purification and Fragmentation of Antibodies, in Current Protocols in Immunology, editors: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, and Strober W. Published by John Wiley & Sons, Inc., New York, 2001).

ELISA assay

Elisa assays were performed as described herein (see Example 2).

Determination of binding affinities

Binding affinities of KFCC-GY4 and KFCC-GY5 anti-PLVAP monoclonal antibodies were measured at the ANT Technology Co., Ltd. (Taipei, Taiwan) using ANTQ300 quartz crystal microbalance technology (Lin S., et al. J Immunol Methods 239:121-124 (2000)).

Isolation and culture of human umbilical vascular endothelial cells (HUVEC)

Isolation and culture of HUVEC were carried out according to the established protocol described in Baudin B, Brunee A, Bosselut N and Vaubourdolle M. Nature Protocols 2:481-485 (2007). During the maintenance of endothelial cell culture, 1% gelatin (DIFCO, Corp.) dissolved in phosphate buffered saline was used to replace collagen solution for coating culture plates or coverslips.

Extraction of hydrophobic membrane proteins of HUVEC by Triton X-114 (TX-114) containing buffer

Five hundred thousand HUVEC were seeded in a 10 cm culture dish for 24 hours. The cells were then stimulated with human VEGF at 40 ng/ml for an additional 72 hours. The cultured cells were washed with 5 ml phosphate buffered saline (PBS) twice. The cells then were detached and lifted from the dish by incubation with 1 ml PBS containing 2 mM EDTA, were placed into a centrifuge tube, and were collected by centrifugation at 300x g for 5 minutes. There were approximately 2 million cells in the pellet produced by centrifugation. The cell pellets were re-suspended in 200 µl ice cold 0.05 M Tris buffer, containing 5 mM EDTA and 0.5% (v/v) Triton X-114 (TX-114), pH 7.4. The solubilized cell suspension was incubated on ice with occasional gentle vortexing. Thereafter, the cells suspension was centrifuged at 10,000x g for 10 minutes at 4°C to remove insoluble cellular debris. The supernatant was transferred to a clean microfuge tube and incubated at 37°C for 5 minutes. During the incubation TX-114 became separated from the aqueous phase. The microfuge tube was then centrifuged at 1000x g for 10 minutes at room temperature, such that the TX-114 was centrifuged to the bottom of the tube. The aqueous phase at the top of the tube was removed and the TX-114 pellet containing hydrophobic cellular proteins was dissolved in 2x SDS acrylamide gel sample buffer in a final volume of 50 µl. Fifteen µl of sample was used for SDS acrylamide gel electrophoresis.

SDS acrylamide gel electrophoresis, preparation of Western blot and immunoblotting

The procedures are the same as previously described by Kao KJ, Scornik JC and McQueen CF. Human Immunol 27:285-297 (1990), with slight modification. Detection of antibody binding on Western blots was carried out using alkaline phosphatase chemiluminescent substrate and an LAS-4000 Luminescent Image Analyzer (Fujifilm Corp.)

Immunofluorescent microscopy

Materials:

1). Primary antibodies:

a.) Normal mouse IgG (Sigma Corp., catalog #:I-5381) dissolved in phosphate buffered saline (PBS) to 1mg/mL as a stock solution, diluted with PBS-0.5% BSA to a concentration of 5 µg/mL before use;

b.) Monoclonal mouse anti-human von Willebrand factor (vWF) (DakoCytomation Corp., catalog#:M0616) diluted 50x with PBS containing 0.5% BSA before use;

c.) Purified KFCC-GY4 and KFCC-GY5 anti-PLVAP monoclonal antibodies were diluted to 5 µg/ml with PBS containing 0.5% BSA before use;

2). Secondary antibody: FITC- conjugated Goat $F(ab')_2$ anti-mouse IgG (H&L) (Serotec, Corp., catalog # : Star105F);

3). VectaShield Mounting Medium with DAPI (*Vector Labs, Corp., catalog* #: H-1200);

4). 100% Methanol (Merck corp. calalog #:1.06009); and

5). Hank's Balanced Salt Solution (HBSS) (Gibco, Corp., catalog#: 12065-056) diluted to 1x before use.

Procedure:

To prepare human umbilical cord vascular endothelial cells for immunofluorescent study, fifty thousand cells were placed in each well of a 24-well culture plate with a 1.5 cm sterile round coverslip placed at the bottom of each well. Each well contained 0.5 ml M199 culture media that was supplemented with 20% fetal calf serum, 1% L-glutamine, 1% antibiotic/antimycotic solution, 50 µg/ml heparin and 75 µg/ml endothelial cell growth supplement (Sigma, Corp. E0760). Each coverslip was pre-coated with 200 µl of 0.4 mg/ml calf skin collagen (Sigma Corp. C9791) in 0.04% acetic acid (v/v) overnight. The coverslips were then washed with sterile 1x phosphate buffered saline (PBS), and subsequently air-dried for use. Cells were cultured overnight and then stimulated with 40 ng/ml vascular endothelial growth factor (VEGF) for an additional 72 hours. The cells on the coverslips were used for the immunofluorescent procedure.

To stain the cells for immunofluorescent microscopy, the cells grown on the coverslip in each well were washed with 0.5 ml 1x HBSS. The cells were then fixed and permeabilized in 0.5 ml ice cold methanol for 5 minutes. The fixed cells were washed 3 times with 0.5 ml 1x PBS for 5 minutes per wash. The fixed cells were then blocked with 0.5 ml 1x PBS containing 0.5% BSA for 1 hour at room temperature. The coverslip containing the fixed cells was removed and placed on top of 0.2 ml diluted primary antibody solution, which contained 5 µg/ml normal IgG, KFCC-GY4 or KFCC-GY5 anti-PLVAP monoclonal antibody, or a 50x dilution of anti-human vWF monoclonal antibody, with the fixed cells facing down and in contact with antibody solution. The antibody solution was placed on a piece of parafilm in a small covered plastic container. The humidity inside was maintained by placing a small piece of filter paper wetted with water.

After incubation at 37°C for one hour in a humidified container, the coverslip was removed and the cells on the coverslip were washed 3 times with 0.5 ml PBS for 5 minutes each time. The fixed cells were then incubated with 0.2 ml 200x-diluted FITC- conjugated Goat $F(ab')_2$ anti-mouse IgG secondary antibody for 50 minutes at 37°C as described for incubation with primary antibody solution. Thereafter, the cells were washed 3 times with PBS as described above. The stained cells were mounted on a glass slide using VectaShield anti-fade solution. Excess mounting media was removed from the edge of the coverslip and the edge was sealed with nail polish. The stained cells were examined using a fluorescent microscope.

Results

Immunization of Balb/cByJ mice with recombinant human PLVAP protein led to the development of hybridomas producing monoclonal antibodies (mAbs) that recognized human PLVAP protein. Two

hybridomas were selected for further study. The antibodies produced by these hybridomas were named KFCC-GY4 and KFCC-GY5. The sequences of the V_H and V_L domains of monoclonal antibodies KFCC-GY4 and KFCC-GY5, and the CDRs of these domains, are shown in FIGS. 15A and B, and 16A and B, respectively.

Both KFCC-GY4 and KFCC-GY5 monoclonal antibodies bound recombinant PLVAP protein in ELISA (FIG. 17) and immunoblot (FIGS. 18C and D) assays.

These antibodies also specifically reacted with PLVAP protein in extracts from human umbilical cord vascular endothelial cells in an immunoblot assay (FIGS. 19B and 19D). In addition, immunofluorescence staining experiments showed binding of KFCC-GY4 and KFCC-GY5 monoclonal antibodies to PLVAP-expressing human vascular endothelial cells (FIGS. 20C and D).

Binding affinities (K_d) of the monoclonal antibodies for recombinant PLVAP protein were determined to be 0.41 x 10^{-7} M for KFCC-GY5 mAb and 0.6 x 10^{-7} M for KFCC-GY4 mAb using ANTQ300 quartz crystal microbalance (Lin, et al. J. Immunol. Methods 239:121-124, 2000).

Immunohistochemistry experiments performed on hepatoma sections from the liver of two different hepatoma patients using KFCC-GY4 or KFCC-GY5 monoclonal anti-PLVAP antibodies showed that the KFCC-GY5 monoclonal antibody produced a stronger signal in vascular endothelial cells (FIGS. 21A, C) than the KFCC-GY4 monoclonal antibody (FIGS. 21B, D).

Immunohistochemistry experiments performed on adjacent hepatoma and non-tumorous liver tissue sections from the liver of the same patient were performed on samples from four different randomly selected hepatoma patients using the KFCC-GY4 monoclonal anti-PLVAP antibody. PLVAP expression was detected in vascular endothelial cells of hepatoma tissues (FIGS. 22A, C, E, and G), but not adjacent non-tumorous liver tissues (FIGS. 22B, D, F, and H).

Example 4: PLVAP protein is expressed on the surfaces of vascular endothelial cells

Materials and methods

Immunofluorescent microscopy

Reagents:

The reagents used for the following procedure are as described in Example 3, with the following modifications:

- the 1x HBSS wash buffer contained 0.1% sodium azide, which was used to prevent endocytosis of antibodies bound to the cell surface.;
- the KFCC-GY4 and KFCC-GY5 monoclonal anti-PLVAP antibodies were diluted in the 1x HBSS wash buffer with 0.1% sodium azide;

Procedure:

Immunofluorescent staining of human umbilical cord vascular endothelial cells (HUVECs) was performed as described in Example 3, except that the cells were not fixed and permeabilized with methanol. Instead, after incubation with anti-PLVAP monoclonal antibodies, the cells were washed and fixed with 4% paraformaldehyde at room temperature for 10 minutes. Following this incubation, the cells were washed 3 times, then were incubated with FITC- conjugated Goat $F(ab')_2$ anti-mouse IgG. After three additional washes, the cells were processed for immunofluorescent microscopy as described in Example 3.

Results

Using the approach described above, only PLVAP protein expressed on the cell surface could be detected. The results of these experiments revealed that both KFCC-GY4 and KFCC-GY5 anti-PLVAP monoclonal antibodies bound to the surface of HCC vascular endothelial cells (FIGS. 23B,C), indicating that PLVAP protein is expressed on the surfaces of these cells. These findings suggest that antibodies that specifically bind PLVAP with high affinity will be able to bind to the surface of HCC vascular endothelial cells upon injection into the blood vessels of a hepatocellular carcinoma tumor.

SEQUENCE LISTING

<110> China Synthetic Rubber Corporation Kao, Kuo-Jang Huang, Andrew T.

<120> METHODS AND AGENTS FOR THE DIAGNOSIS AND TREATMENT OF HEPATOCELLULAR CARCINOMA

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caggcccggg aggccaagct ccaagctgaa tgctcccggc agacccagct agcgctggag 960
gagaaggcgg tgctgcggaa ggaacgagac aacctggcca aggagctgga agagaagaag 1020
agggaggcgg agcagctcag gatggagctg gccatcagaa actcagccct ggacacctgc 1080
atcaagacca agtcgcagcc gatgatgcca gtgtcaaggc ccatgggccc tgtccccaac 1140
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aggccccctg caggcatccc tgtagcccca tccagtggct gaggaggctc caggcctgag 1260
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tc                                                                1322
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<210> 2

< 211> 413

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> Recombinant His-tagged human PLVAP amino acid residues 51-442

<400> 2

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

Arg Asn Ser Ala Leu Asp Thr Cys Ile Lys Thr Lys Ser Gln Pro Met
        355                 360                 365
Met Pro Val Ser Arg Pro Met Gly Pro Val Pro Asn Pro Gln Pro Ile
    370                 375                 380
Asp Pro Ala Ser Leu Glu Glu Phe Lys Arg Lys Ile Leu Glu Ser Gln
385                 390                 395                 400
Arg Pro Pro Ala Gly Ile Pro Val Ala Pro Ser Ser Gly
                405                 410
```

<210> 3

< 211> 342

< 212> DNA

< 213> Artificial Sequence

<220>

< 223> KFCC-GY4 VH domain coding sequence

<400> 3

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gaggttcagc tgcagcagtc tggggcagag tttgtgaggt caggggcctc agtcaagttg 60
tcctgcacag cttctggctt caacattaaa gactactata tacactgggt gaagcagagg 120
cctgaacagg gcctggagtg gattggatgg attgatcctg agaatggtga tattgaatat 180
gccccgaagt tccagggcaa ggccactatg actgcagaca catcctccaa tacagcctac 240
ctgcagttca gcagcctgac atctgaggac actgccgtct attactgtct ctaccaagaa 300
ggctcctggg gccaaggcac cactctcaca gtctcctcag cc                     342
```

<210> 4

< 211> 114

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> KFCC-GY4 VH domain

<400> 4

```
Glu Val Gln Leu Gln Gln Ser Gly Ala Glu Phe Val Arg Ser Gly Ala
 1               5                  10                  15
Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys Asp Tyr
            20                  25                  30
Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile
        35                  40                  45
Gly Trp Ile Asp Pro Glu Asn Gly Asp Ile Glu Tyr Ala Pro Lys Phe
    50                  55                  60
Gln Gly Lys Ala Thr Met Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr
65                  70                  75                  80
Leu Gln Phe Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Leu Tyr Gln Glu Gly Ser Trp Gly Gln Gly Thr Thr Leu Thr Val Ser
            100                 105                 110
Ser Ala
```

<210> 5

< 211> 5

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR1 sequence

<400> 5

```
Asp Tyr Tyr Ile His
 1               5
```

<210> 6

< 211> 17

< 212> PRT

< 213> CDR2 sequence

<400> 6

```
Trp Ile Asp Pro Glu Asn Gly Asp Ile Glu Tyr Ala Pro Lys Phe Gln
 1               5                  10                  15
Gly
```

<210> 7

< 211> 4

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR3 sequence

<400> 7

Gln Glu Gly Ser
1

<210> 8

< 211> 336

< 212> DNA

< 213> Artificial Sequence

<220>

< 223> KFCC-GY4 VL domain coding sequence

<400> 8

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atctcttgca agtcaagtca gagcctctta aatagtgatg gaaagacata tttgaattgg 120
ttgttacaga ggccaggcca gtctccaaag cgcctaatct atctggtgtc taaattggac 180
tctggagtcc ctgacaggtt cactggcagt ggatcaggga cagatttcac actgaaaatc 240
agcagagtgg aggctgagga tttgggagtt tattattgct ggcaaggtac acattttccg 300
ttcacgttcg gaggggggac caagctggaa ataaaa                           336
```

<210> 9

< 211> 111

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> KFCC-GY4 VL domain

<400> 9

```
Asp Val Val Met Thr Gln Pro Leu Thr Leu Ser Val Thr Ile Gly Gln
 1               5                  10                  15
Pro Ala Ser Ile Ser Cys Lys Ser Ser Gln Ser Leu Leu Asn Ser Asp
            20                  25                  30
Gly Lys Thr Tyr Leu Asn Trp Leu Leu Gln Arg Pro Gly Gln Ser Pro
        35                  40                  45
Lys Arg Leu Ile Tyr Leu Val Ser Lys Leu Asp Ser Gly Val Pro Asp
    50                  55                  60
Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser
65                  70                  75                  80
Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Trp Gln Gly Thr
                85                  90                  95
His Phe Pro Phe Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110
```

<210> 10

< 211> 16

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR1 sequence

<400> 10

Lys Ser Ser Gln Ser Leu Leu Asn Ser Asp Gly Lys Thr Tyr Leu Asn
1 5 10 15

<210> 11

< 211> 7

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR2 sequence

<400> 11

Leu Val Ser Lys Leu Asp Ser
1 5

<210> 12

< 211> 9

< 212> PRT

< 213> CDR3 sequence

<400> 12

Trp Gln Gly Thr His Phe Pro Phe Thr
1 5

<210> 13

< 211> 342

< 212> DNA

< 213> Artificial Sequence

<220>

< 223> KFCC-GY-5-VH domain coding sequence

<400> 13

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tcctgcaagg cttctggcta caccttcacc agcaactaca taaactgggt gaaacagagg 120
cctggacagg gccttgagtg gatcggaaat atttatcctt ctgatggttt tactaactac 180
aatcaaaagt tcaaggacag ggccacattg actgtagaca aatcctccag cacagcctac 240
atgcagctca gcagcccgac atctgaggac tctgcggtct attactgtac aagaaacttc 300
gatgtctggg gcgcagggac cacggtcacc gtctcctcag cc 342

<210> 14

< 211> 114

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> KFCC-GY5 VH domain

<400> 14

```
Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Arg Pro Gly Ala
 1               5                  10                  15
Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Asn
            20                  25                  30
Tyr Ile Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45
Gly Asn Ile Tyr Pro Ser Asp Gly Phe Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60
Lys Asp Arg Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80
Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95
Thr Arg Asn Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val Ser
            100                 105                 110
Ser Ala
```

<210> 15

< 211> 5

< 212> PRT

< 213> CDR1 Sequence

<400> 15

```
Ser Asn Tyr Ile Asn
 1               5
```

<210> 16

< 211> 17

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR2 sequence

<400> 16

```
Asn Ile Tyr Pro Ser Asp Gly Phe Thr Asn Tyr Asn Gln Lys Phe Lys
 1               5                  10                  15
Asp
```

<210> 17

< 211> 4

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR3 sequence

<400> 17

Asn Phe Asp Val

1

<210> 18

< 211> 336

< 212> DNA

< 213> Artificial Sequence

<220>

< 223> KFCC-GY5 VL domain coding sequence

<400> 18

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atctcttgca gatctagtca gagccttgtc cacagtaatg gaaacaccta tttacagtgg 120
tacctgcaga agccaggcca gtctccaaag ctcctgatct acacagtttc caaccgattt 180
tctggggtcc cagacaggtt cagtggcagt ggatcagggc cagatttcac actcaagatc 240
agcagagtgg aggctgagga tctgggagtt tatttctgct ctcaaagtac acatgttcct 300
ttcacgttcg gctcggggac aaagttggaa ataaaa                           336
```

<210> 19

< 211> 112

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> KFCC-GY5 VL domain

<400> 19

```
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
 1               5                  10                  15
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30
Asn Gly Asn Thr Tyr Leu Gln Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45
Pro Lys Leu Leu Ile Tyr Thr Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Pro Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                85                  90                  95
Thr His Val Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110
```

<210> 20

< 211> 16

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR1 sequence

<400> 20

```
Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr Leu Gln
 1               5                  10                  15
```

<210> 21

< 211> 7

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR2 sequence

<400> 21

Thr Val Ser Asn Arg Phe Ser
1 5

<210> 22

< 211> 9

< 212> PRT

< 213> Artificial Sequence

<220>

< 223> CDR3 sequence

<400> 22

Ser Gln Ser Thr His Val Pro Phe Thr
1 5

<210> 23

< 211> 442

< 212> PRT

< 213> Homo sapiens

<400> 23

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

```
                325                 330                 335
Gln Ala Glu Cys Ser Arg Gln Thr Gln Leu Ala Leu Glu Glu Lys Ala
            340                 345                 350
Val Leu Arg Lys Glu Arg Asp Asn Leu Ala Lys Glu Leu Glu Glu Lys
        355                 360                 365
Lys Arg Glu Ala Glu Gln Leu Arg Met Glu Leu Ala Ile Arg Asn Ser
    370                 375                 380
Ala Leu Asp Thr Cys Ile Lys Thr Lys Ser Gln Pro Met Met Pro Val
385                 390                 395                 400
Ser Arg Pro Met Gly Pro Val Pro Asn Pro Gln Pro Ile Asp Pro Ala
                405                 410                 415
Ser Leu Glu Glu Phe Lys Arg Lys Ile Leu Glu Ser Gln Arg Pro Pro
            420                 425                 430
Ala Gly Ile Pro Val Ala Pro Ser Ser Gly
        435                 440
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<210> 24

< 211> 2317

< 212> DNA

< 213> Homo sapiens

<400> 24

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ccatggagca cggagggtcc tacgctcggg cgggggcag ctctcggggc tgctggtatt 120
acctgcgcta cttcttcctc ttcgtctccc tcatccaatt cctcatcatc ctggggctcg 180
tgctcttcat ggtctatggc aacgtgcacg tgagcacaga gtccaacctg caggccaccg 240
agcgccgagc cgagggccta tacagtcagc tcctagggct cacggcctcc cagtccaact 300
tgaccaagga gctcaacttc accacccgcg ccaaggatgc catcatgcag atgtggctga 360
atgctcgccg cgacctggac cgcatcaatg ccagcttccg ccagtgccag ggtgaccggg 420
tcatctacac gaacaatcag aggtacatgg ctgccatcat cttgagtgag aagcaatgca 480
gagatcaatt caaggacatg aacaagagct gcgatgcctt gctcttcatg ctgaatcaga 540
aggtgaagac gctggaggtg gagatagcca aggagaagac catttgcact aaggataagg 600
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gggagctgca gcaccaagag cgccagctgg ccaaggagca actgcaaaag gtgcaagccc 720
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ccattatccc acgcagcctg gacaacctgg gttacaacct ctaccatccc ctgggctcgg 840
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aggagctggc ccggagcctc cgggcggata tcgaacgcgt ggcccgcgag aactcagacc 960
tccaacgcca gaagctggaa gcccagcagg gcctgcgggc cagtcaggag gcgaaacaga 1020
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agctggaaga gaagaagagg gaggcggagc agctcaggat ggagctggcc atcagaaact 1200
cagccctgga cacctgcatc aagaccaagt cgcagccgat gatgccagtg tcaaggccca 1260
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cgatccctca cccagagcac acggccgcgg agatgacgtc acgcaagcaa cggcgctgac 1620
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acaccgagat gcagcaacga cgtcacgggc catgtcgacg tcacacatat taatgtcaca 1800
cagacgcggc gatggcatca cacagacggt gatgatgtca cacacagaca cagtgacaac 1860

acacaccatg acaacgacac ctatagatat ggcaccaaca tcacatgcac gcatgcccct 1920
tcacacacac tttctaccca attctcacct agtgtcacgt tcccccgacc ctggcacacg 1980
ggccaaggta cccacaggat cccatcccct cccgcacagc cctgggcccc agcacctccc 2040
ctcctccagc ttcctggcct cccagccact tcctcacccc cagtgcctgg acccggaggt 2100
gagaacagga agccattcac ctccgctcct tgagcgtgag tgtttccagg acccccctcgg 2160
ggccctgagc cgggggtgag ggtcacctgt tgtcgggagg ggagccactc cttctccccc 2220
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atccttaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaa                          2317
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<210> 25

< 211> 18

< 212> DNA

< 213> Artificial Sequence

<220>
< 223> oligonucleotide primer

<400> 25
cctgcaggca tccctgta 18

<210> 26
< 211> 16
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide primer

<400> 26
cgggccatcc cttggt 16

<210> 27
< 211> 16
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide probe

<400> 27
ccccatccag tggctg 16

<210> 28
< 211> 21
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide primer

<400> 28
ccttgagcgt gagtgtttcc a 21

<210> 29
< 211> 25
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide primer

<400> 29
gtcccccaac ttgagatgta tgaag 25

<210> 30
< 211> 17
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide primer

<400> 30
ggcagggctg ggagttg 17

<210> 31
< 211> 25
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide primer

<400> 31
gtctcaagtc agtgtacagg taagc 25

<210> 32
< 211> 16
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide probe

<400> 32
ctcccaggga gaccaa 16

<210> 33
< 211> 18
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide probe

<400> 33
aaggagtggc tcccctcc 18

<210> 34
< 211> 30
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide primer

<400> 34
catatgaacg tgcacgtgag cacagagtcc 30

<210> 35
< 211> 28
< 212> DNA
< 213> Artificial Sequence

<220>
< 223> oligonucleotide primer

<400> 35
ggatcctgag catatccctg catcctcc 28

Patentkrav

1. Antistof eller antigen-bindende fragment deraf der specifikt binder SEQ ID NO: 23 med en K_d på 10^{-7} M eller derunder som målt under anvendelse af en ANTQ300 kvartskrystal mikrovægt, til anvendelse i en in vivo-fremgangsmåde til detektering af hepatocellulært carcinom (HCC) i et individ omfattende administrering af antistoffet ved intraarteriel injektion eller intravenøs injektion, hvor antistoffet omfatter en radioisotop, givende et billede af leveren af et individ og detektion af akkumuleringen af antistoffet i leveren på et individ hvor antistoffet eller det antigenbindingende fragment omfatter

a) et antistofvariabelt domæne omfattende CDR1 bestående af SEQ ID NO:5, CDR2 bestående af SEQ ID NO:6, og CDR3 bestående af SEQ ID NO:7; og et antistofvariabelt domæne omfattende CDR1 bestående af SEQ ID NO:10, CDR2 bestående af SEQ ID NO:11, og CDR3 bestående af SEQ ID NO:12; eller

b) et antistofvariabelt domæne omfattende CDR1 bestående af SEQ ID NO:15, CDR2 bestående af SEQ ID NO:16, og CDR3 bestående af SEQ ID NO:17 og et antistofvariabelt domæne omfattende CDR1 bestående af SEQ ID NO:20, CDR2 bestående af SEQ ID NO:21, og CDR3 bestående af SEQ ID NO:22.

2. Antistoffet eller det antigenbindende fragment deraf til anvendelse i krav 1 (a), hvor antistoffet eller det antigenbindende fragment deraf omfatter et V_H-domæne med aminosyresekvensen af SEQ ID NO: 4.

3. Antistoffet eller det antigenbindende fragment deraf til anvendelse i krav 1 (a), eller krav 2, hvor antistoffet omfatter en V_L-domæne med aminosyresekvensen af SEQ ID NO: 9.

4. Antistoffet eller det antigenbindende fragment deraf til anvendelse i krav 1 (b), hvor antistoffet eller det antigenbindende fragment deraf omfatter et V_H-domæne med aminosyresekvensen af SEQ ID NO: 14.

5. Antistoffet eller det antigenbindende fragment deraf til anvendelse i krav 1 (b), eller krav 4, hvor antistoffet eller det antigenbindende fragment deraf omfatter et V_L-domæne med aminosyresekvensen af SEQ ID NO: 19.

6. Antistoffet eller det antigenbindende fragment deraf til anvendelse af et hvilket som helst af de foregående krav, hvor antistoffet er et monoklonalt antistof, et polyklonalt antistof, et kimært antistof, et humaniseret antistof, eller et humant antistof.

7. Fremgangsmåde til diagnosticering af et hepatocellulært carcinom i et individ omfattende detektion af niveauet af PLVAP-protein i en prøve fra individet og bestemmelse af at niveauet af PLVAP-proteinet i prøven er steget i forhold til en kontrol, hvor niveauet af PLVAP-protein i en prøve fra individet detekteres under anvendelse af et antistof eller antigenbindende fragment deraf der omfatter

a) et antistofvariabelt domæne omfattende CDR1 bestående af SEQ ID NO:5, CDR2 bestående af SEQ ID NO:6, og CDR3 bestående af SEQ ID NO:7; og et antistofvariabelt domæne omfattende CDR1 bestående af SEQ ID NO:10, CDR2 bestående af SEQ ID NO: 11, og CDR3 bestående af SEQ ID NO:12; eller

b) et antistofvariabelt domæne omfattende CDR1 bestående af SEQ ID NO:15, CDR2 bestående af SEQ ID NO:16, og CDR3 bestående af SEQ ID NO:17 og et antistofvariabelt domæne omfattende CDR1 bestående af SEQ ID NO:20, CDR2 bestående af SEQ ID NO:21, og CDR3 bestående af SEQ ID NO:22.

8. Fremgangsmåden af krav 7 (a), hvor antistoffet eller det antigenbindende fragment deraf omfatter et V_H-domæne med aminosyresekvensen af SEQ ID NO: 4.

9. Fremgangsmåden af krav 7 (a), eller krav 8, hvor antistoffet omfatter et V_L-domæne med aminosyresekvensen af SEQ ID NO: 9.

10. Fremgangsmåden af krav 7 (b), hvor antistoffet eller det antigenbindende fragment deraf omfatter et V_H-domæne med aminosyresekvensen af SEQ ID NO: 14.

11. Fremgangsmåden af krav 7 (b), eller krav 10, hvor antistoffet eller det antigenbindende fragment deraf omfatter et V_L-domæne med aminosyresekvensen af SEQ ID NO: 19.

12. Fremgangsmåden ifølge et hvilket som helst af kravene 7 til 11, hvor antistoffet er et monoklonalt antistof, et polyklonalt antistof, et kimært antistof, et humaniseret antistof, eller et humant antistof.

13. Fremgangsmåden ifølge et hvilket som helst af kravene 7 til 12, hvor antistoffet eller det antigenbindende fragment deraf er mærket med en radioaktiv isotop.

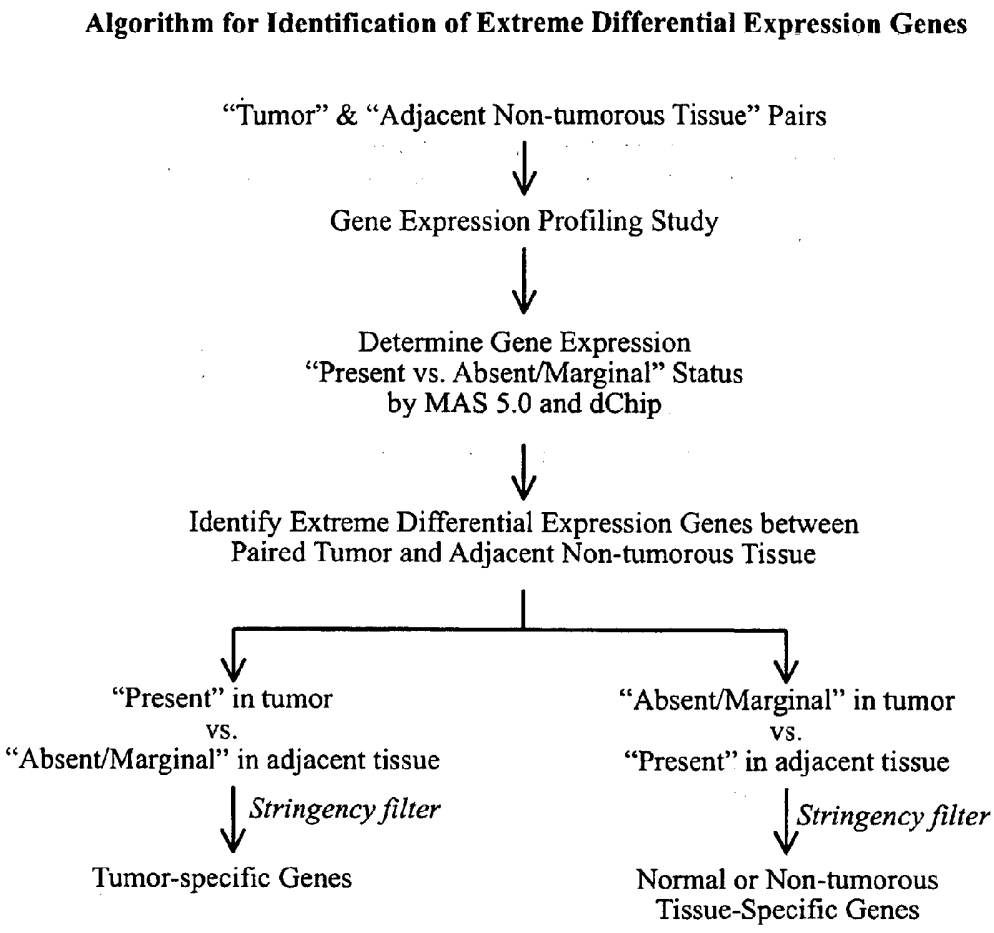

Algorithm for Identification of Extreme Differential Expression Genes
"Tumor" & "Adjacent Non-tumorous Tissue" Pairs
Gene Expression Profiling Study
Determine Gene Expression
"Present vs. Absent/Marginal" Status
by MAS 5.0 and dChip
Identify Extreme Differential Expression Genes between
Paired Tumor and Adjacent Non-tumorous Tissue
"Present" in tumor
vs.
"Absent/Marginal" in adjacent tissue
Stringency filter
Tumor-specific Genes
"Absent/Marginal" in tumor
vs.
"Present" in adjacent tissue
Stringency filter
Normal or Non-tumorous
Tissue-Specific Genes

FIG. 1

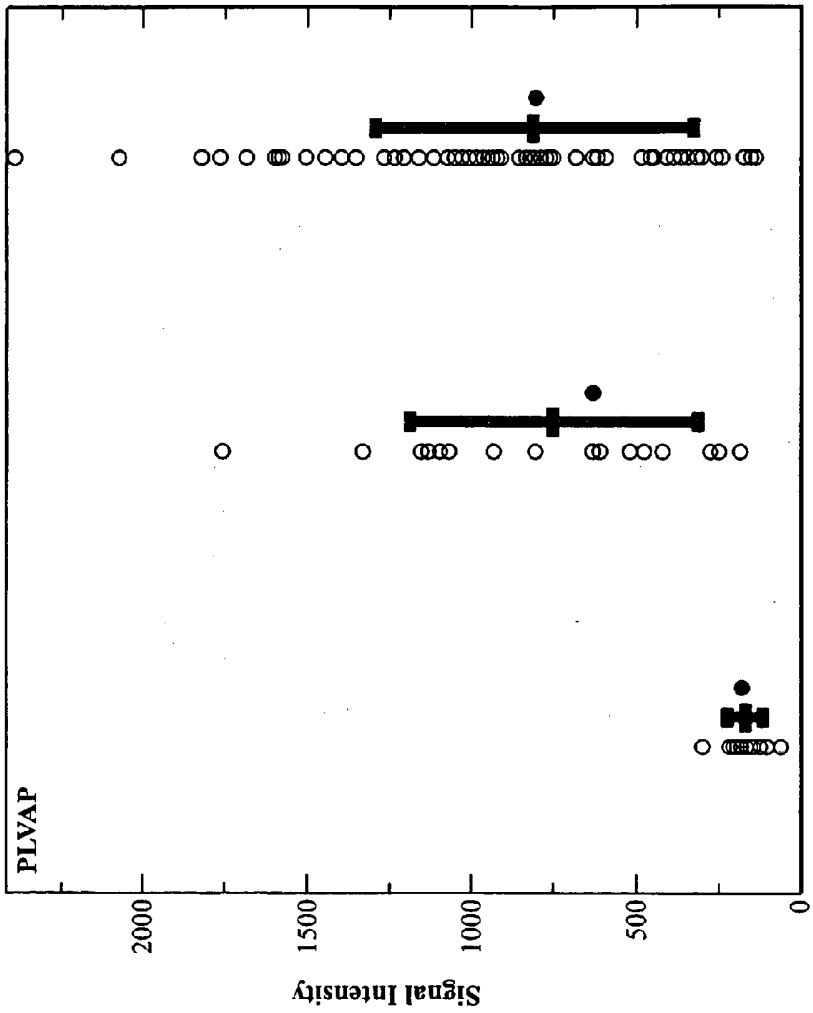


FIG. 2

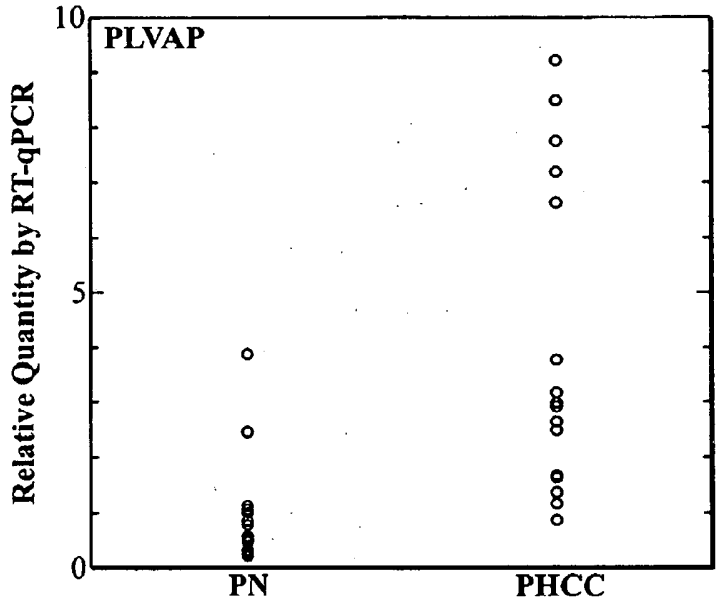
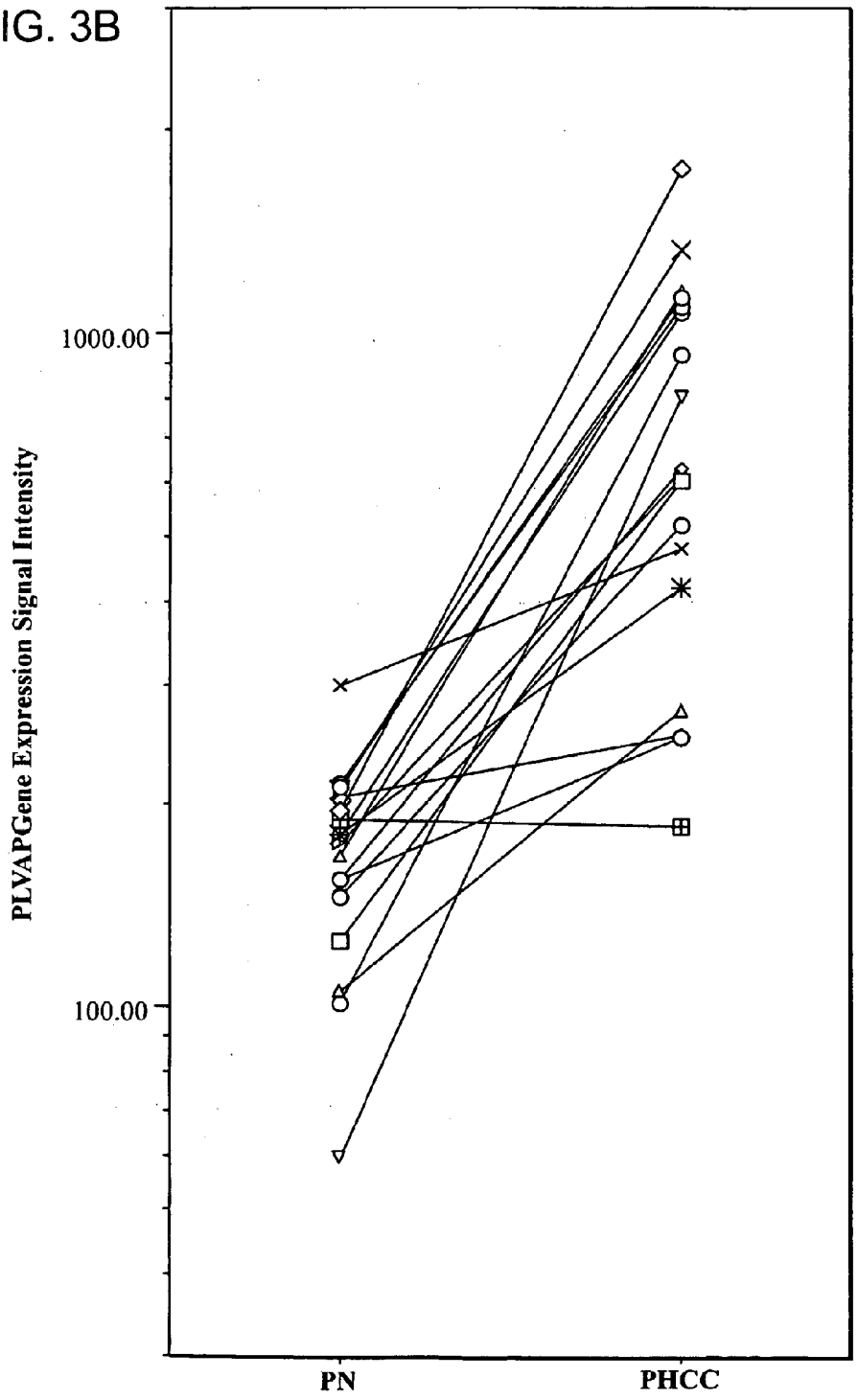


FIG. 3A

FIG. 3B


PLVAPGene Expression Signal Intensity
1000.00
100.00
PN
PHCC

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  1 atgggcagcagccatcatcatcatcacagcagcggcctggtg
    M  G  S  S  H  H  H  H  H  H  S  S  G  L  V
 46 ccgcgcggcagccatatgaacgtgcacgtgagcacagagtccaac
    P  R  G  S  H  M  N  V  H  V  S  T  E  S  N
 91 ctgcaggccaccgagcgccgagccgagggcctatacagtcagctc
    L  Q  A  T  E  R  R  A  E  G  L  Y  S  Q  L
136 ctagggctcacggcctcccagtccaacttgaccaaggagctcaac
    L  G  L  T  A  S  Q  S  N  L  T  K  E  L  N
181 ttcaccacccgcgccaaggatgccatcatgcagatgtggctgaat
    F  T  T  R  A  K  D  A  I  M  Q  M  W  L  N
226 gctcgccgcgacctggaccgcatcaatgccagcttccgccagtgc
    A  R  R  D  L  D  R  I  N  A  S  F  R  Q  C
271 cagggtgaccgggtcatctacacgaacaatcagaggtacatggct
    Q  G  D  R  V  I  Y  T  N  N  Q  R  Y  M  A
316 gccatcatcttgagtgagaagcaatgcagagatcaattcaaggac
    A  I  I  L  S  E  K  Q  C  R  D  Q  F  K  D
361 atgaacaagagctgcgatgccttgctcttcatgctgaatcagaag
    M  N  K  S  C  D  A  L  L  F  M  L  N  Q  K
406 gtgaagacgctggaggtggagatagccaaggagaagaccatttgc
    V  K  T  L  E  V  E  I  A  K  E  K  T  I  C
451 actaaggataaggaaagcgtgctgctgaacaaacgcgtggcggag
    T  K  D  K  E  S  V  L  L  N  K  R  V  A  E
496 gaacagctggttgaatgcgtgaaaacccgggagctgcagcaccaa
    E  Q  L  V  E  C  V  K  T  R  E  L  Q  H  Q
541 gagcgccagctggccaaggagcaactgcaaaaggtgcaagccctc
    E  R  Q  L  A  K  E  Q  L  Q  K  V  Q  A  L
586 tgcctgcccctggacaaggacaagtttgagatggaccttcgtaac
    C  L  P  L  D  K  D  K  F  E  M  D  L  R  N
631 ctgtggagggactccattatcccacgcagcctggacaacctgggt
    L  W  R  D  S  I  I  P  R  S  L  D  N  L  G
676 tacaacctctaccatcccctgggctcggaattggcctccatccgc
    Y  N  L  Y  H  P  L  G  S  E  L  A  S  I  R
721 agagcctgcgaccacatgcccagcctcatgagctccaaggtggag
    R  A  C  D  H  M  P  S  L  M  S  S  K  V  E
766 gagctggcccggagcctccgggcggatatcgaacgcgtggcccgc
    E  L  A  R  S  L  R  A  D  I  E  R  V  A  R
811 gagaactcagacctccaacgccagaagctggaagcccagcagggc
    E  N  S  D  L  Q  R  Q  K  L  E  A  Q  Q  G
856 ctgcgggccagtcaggaggcgaaacagaaggtggagaaggaggct
    L  R  A  S  Q  E  A  K  Q  K  V  E  K  E  A
901 caggcccgggaggccaagctccaagctgaatgctcccggcagacc
    Q  A  R  E  A  K  L  Q  A  E  C  S  R  Q  T
946 cagctagcgctggaggagaaggcggtgctgcggaaggaacgagac
    Q  L  A  L  E  E  K  A  V  L  R  K  E  R  D
```

FIG. 4A

```
 991 aacctggccaaggagctggaagagaagaagagggaggcggagcag
     N  L  A  K  E  L  E  E  K  K  R  E  A  E  Q
1036 ctcaggatggagctggccatcagaaactcagccctggacacctgc
     L  R  M  E  L  A  I  R  N  S  A  L  D  T  C
1081 atcaagaccaagtcgcagccgatgatgccagtgtcaaggcccatg
     I  K  T  K  S  Q  P  M  M  P  V  S  R  P  M
1126 ggccctgtccccaacccccagcccatcgacccagctagcctggag
     G  P  V  P  N  P  Q  P  I  D  P  A  S  L  E
1171 gagttcaagaggaagatcctggagtcccagaggccccctgcaggc
     E  F  K  R  K  I  L  E  S  Q  R  P  P  A  G
1216 atccctgtagccccatccagtggctga
     I  P  V  A  P  S  S  G  *  (SEQ ID NO:2)
   ggaggctccaggcctgaggaccaagggatggcccgactcggcggt

   ttgcggaggatgcagggatatgctcacaggattc(SEQ ID NO:1)
```

FIG. 4B

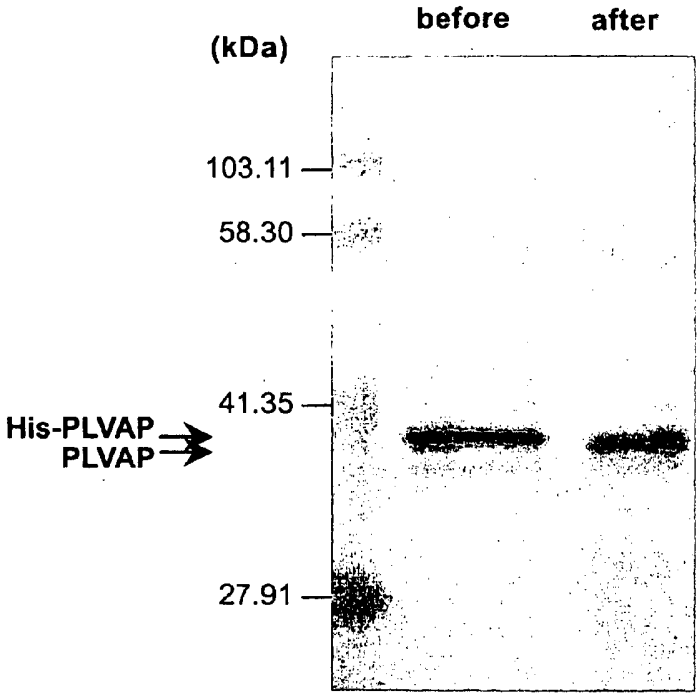


FIG. 5

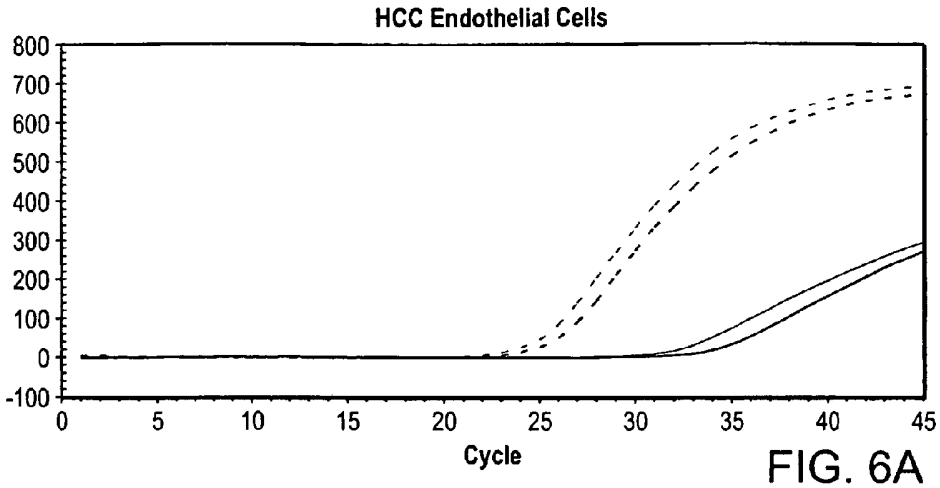


FIG. 6A

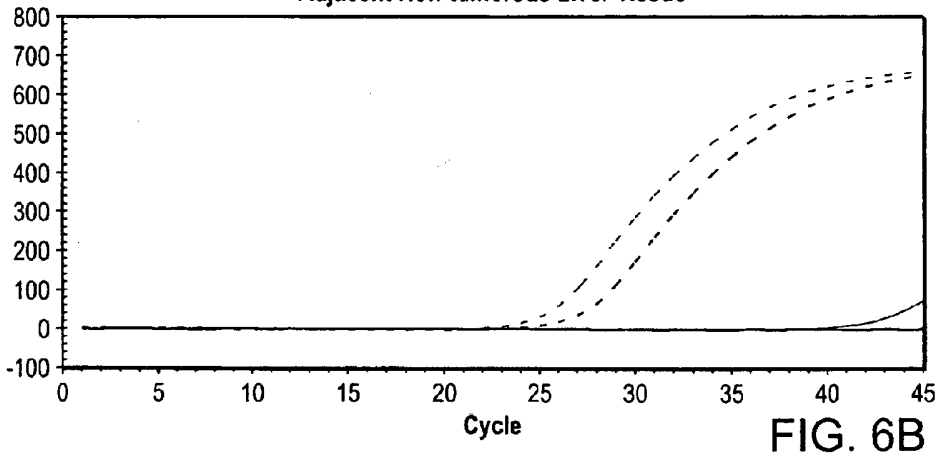


FIG. 6B

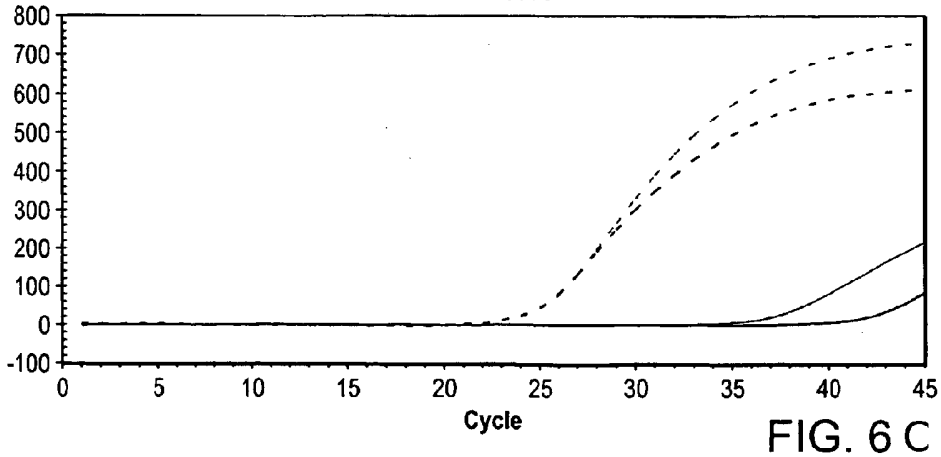


FIG. 6 C

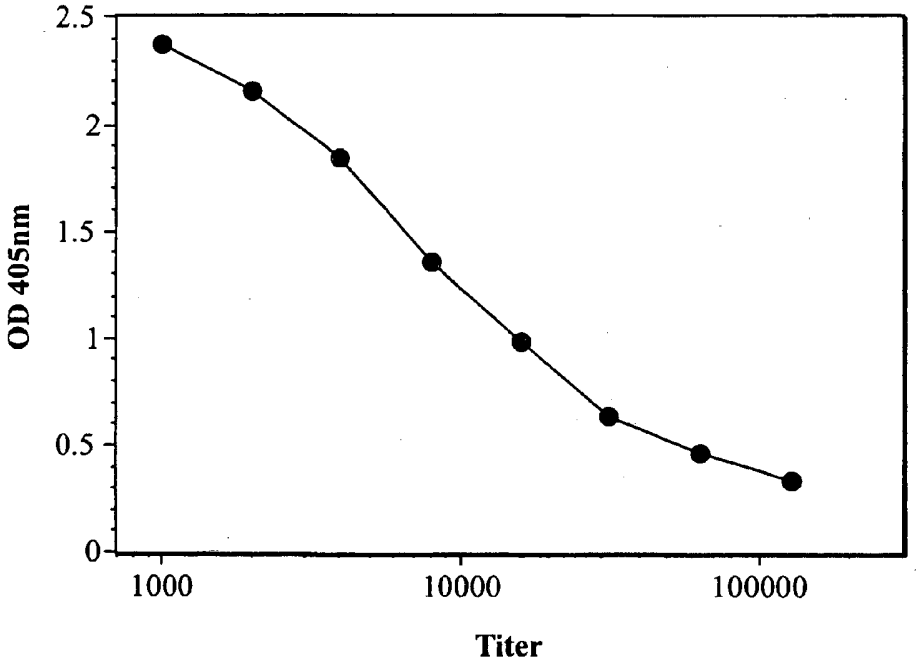


FIG. 7

FIG. 8B

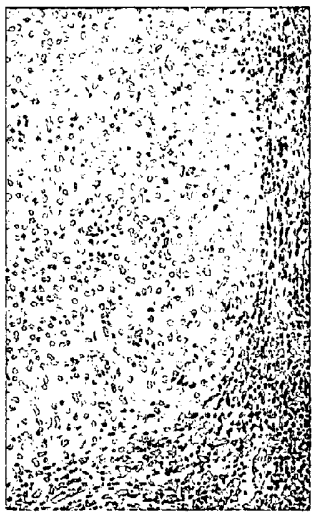


FIG. 8D

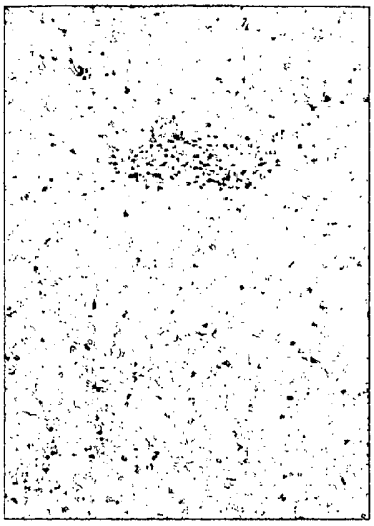


FIG. 8F

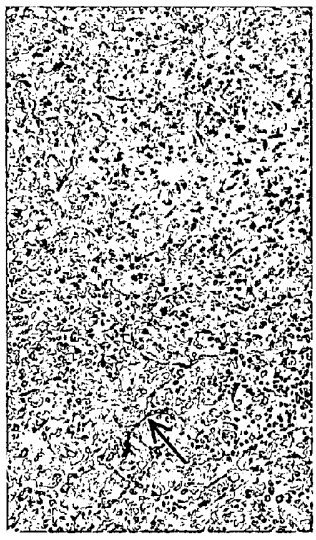


FIG. 8A

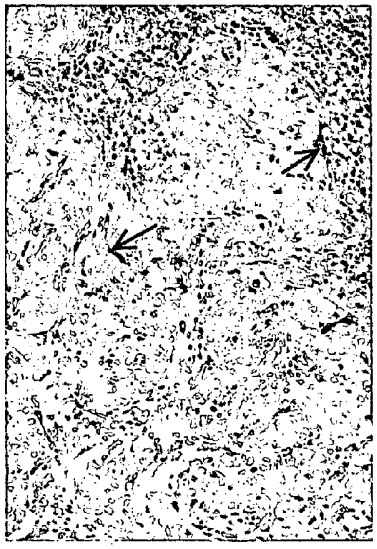


FIG. 8C

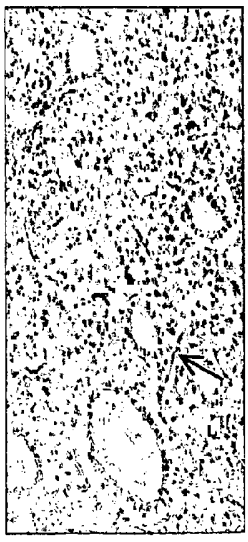


FIG. 8E

FIG. 9A

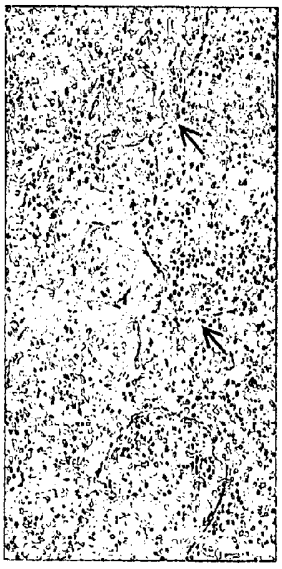


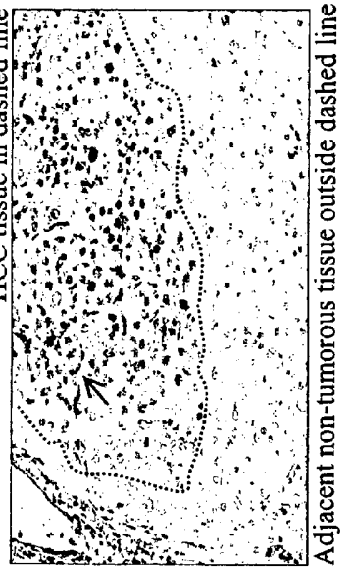
FIG. 9B

FIG. 9C

FIG. 9D

FIG. 9E

FIG. 9F



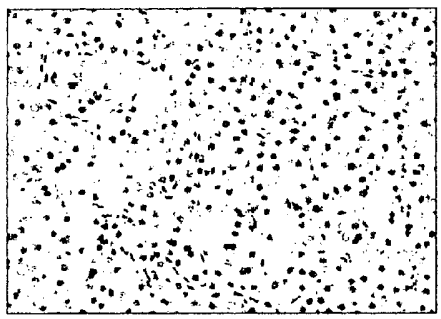


FIG. 10C

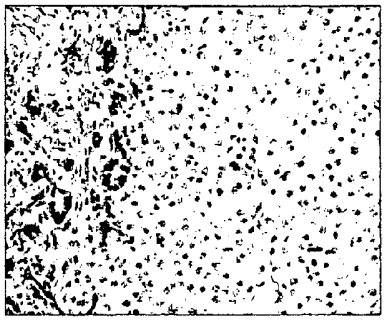


FIG. 10F

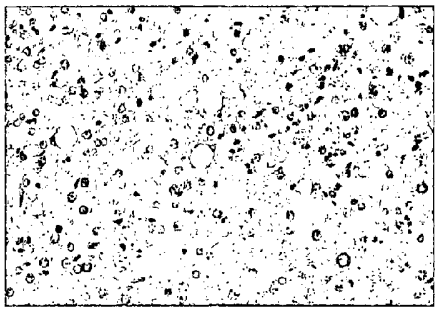


FIG. 10B

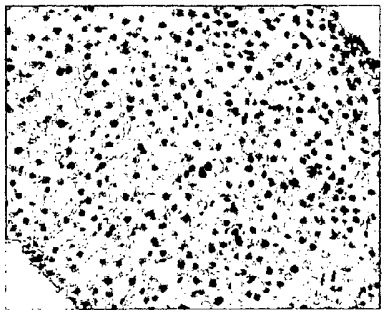


FIG. 10E

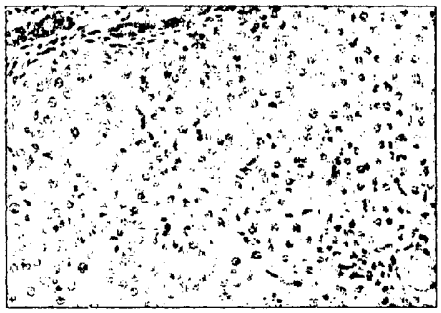


FIG. 10A

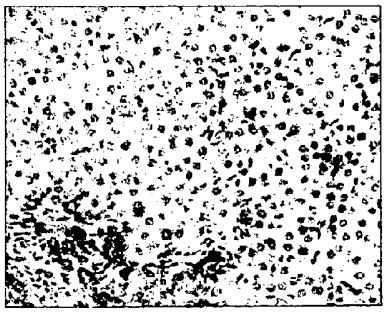


FIG. 10D

FIG. 11B

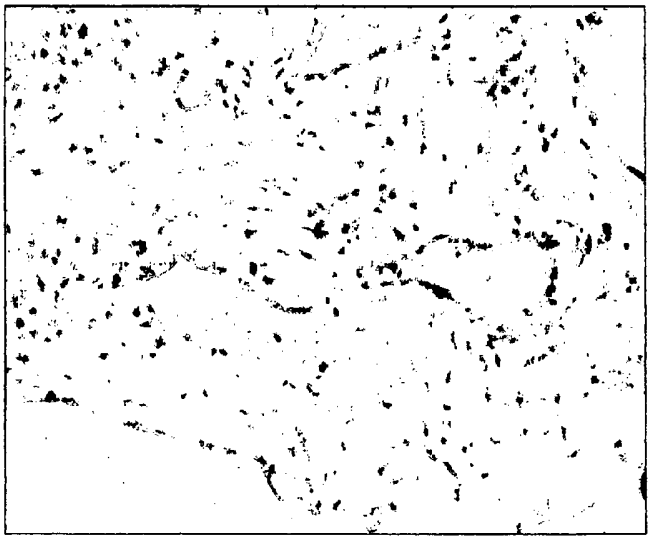


FIG. 11A

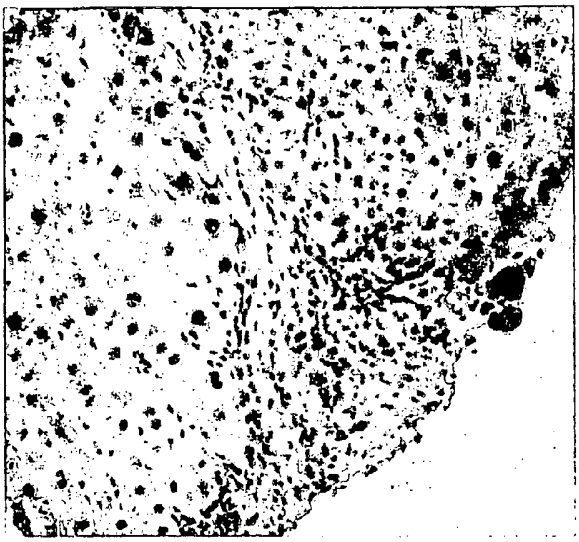


FIG. 12B

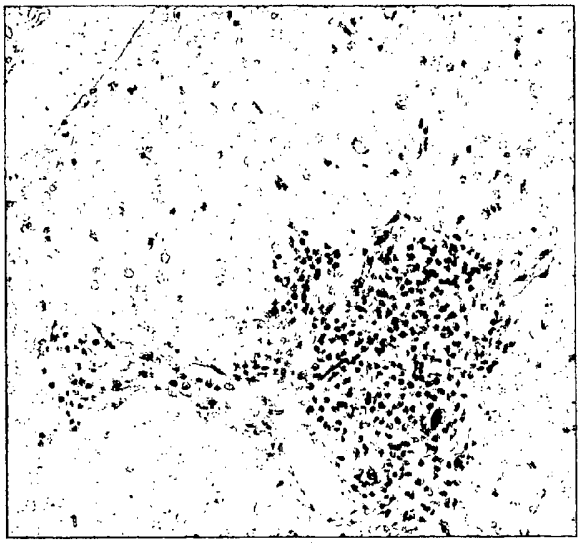


FIG. 12A

FIG. 13A

FIG. 13B

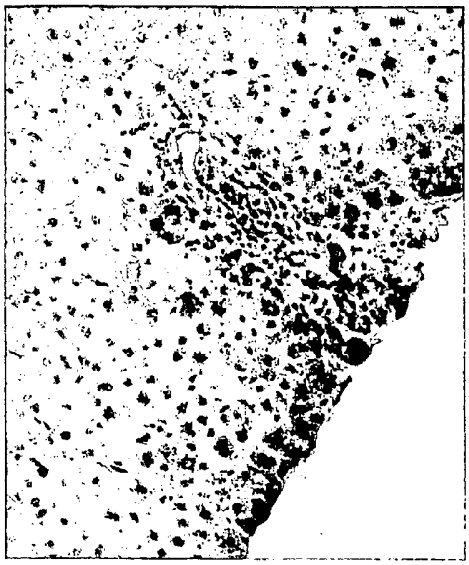


FIG. 13C

FIG. 13D

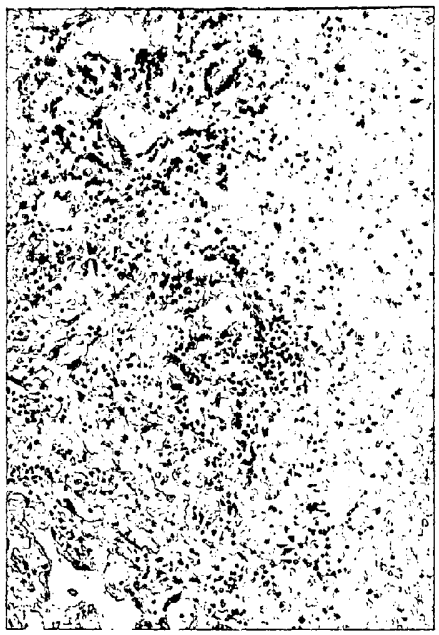


FIG. 14B

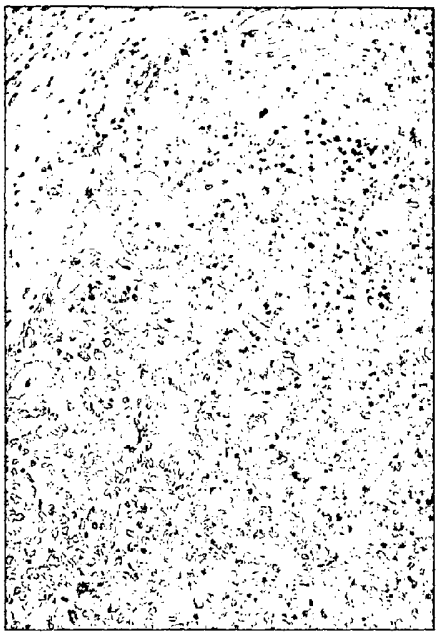


FIG. 14D

FIG. 14A

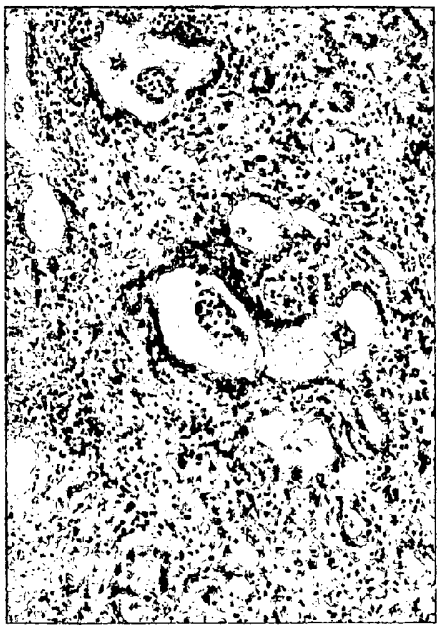


FIG. 14C

GAGGTTCAGCTGCAGCAGTCTGGGGCAGAGTTTGTGAGGTCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTTCTGGCTTCAACATTAAA 90
GACTACTATATACACTGGGTGAAGCAGAGGCCTGAACAGGGCCTGGAGTGGATTGGATGGATTGATCCTGAGAATGGTGATATTGAATAT 180
GCCCCGAAGTTCCAGGGCAAGGCCACTATGACTGCAGACACATCCTCCAATACAGCCTACCTGCAGTTCAGCAGCCTGACATCTGAGGAC 270
ACTGCCGTCTATTACTGTCTCTACCAAGAAGGCTCCTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCC 342 (SEQ ID NO:3)

E V Q L Q Q S G A E F V R S G A S V K L S C T A S G F N I K
D Y Y I H W V K Q R P E Q G L E W I G W I D P E N G D I E Y
CDR1 CDR2
A P K F Q G K A T M T A D T S S N T A Y L Q F S S L T S E D
T A V Y Y C L Y Q E G S W G Q G T T L T V S S A (SEQ ID NO:4)
CDR3

CDR1: D Y Y I H (SEQ ID NO:5)
CDR2: W I D P E N G D I E Y A P K F Q G (SEQ ID NO:6)
CDR3: Q E G S (SEQ ID NO:7)

FIG. 15A

GATGTTGTGATGACCCAGACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCCATCTCTTGCAAGTCAAGTCAGAGCCTCTTA 90
AATAGTGATGGAAAGACATATTTGAATTGGTTGTTACAGAGGCCAGGCCAGTCTCCAAAGCGCCTAATCTATCTGGTGTCTAAATTGGAC 180
TCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCAGGGACAGATTTCACACTGAAAATCAGCAGAGTGGAGGCTGAGGATTTGGGAGTT 270
TATTATTGCTGGCAAGGTACACATTTTCCGTTCACGTTCGGAGGGGGGACCAAGCTGGAAATAAAA 336 (SEQ ID NO:8)

D V V M T Q T P L T L S V T I G Q P A S I S C K S S Q S L L
N S D G K T Y L N W L L Q R P G Q S P K R L I Y L V S K L D
CDR1 CDR2
S G V P D R F T G S G S G T D F T L K I S R V E A E D L G V
Y Y C W Q G T H F P F T F G G G T K L E I K (SEQ ID NO:9)
CDR3

CDR1: K S S Q S L L N S D G K T Y L N (SEQ ID NO:10)
CDR2: L V S K L D S (SEQ ID NO:11)
CDR3: W Q G T H F P F T (SEQ ID NO:12)

FIG. 15B

CAGGTCCAACTGCAGCAGCCTGGGGCTGAGCTGGTGAGGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCTTCTGGCTACACCTTCACC 90
AGCAACTACATAAACTGGGTGAAACAGAGGCCTGGACAGGGCCTTGAGTGGATCGGAAATATTTATCCTTCTGATGGTTTTACTAACTAC 180
AATCAAAAGTTCAAGGACAGGGCCACATTGACTGTAGACAAATCCTCCAGCACAGCCTACATGCAGCTCAGCAGCCCGACATCTGAGGAC 270
TCTGCGGTCTATTACTGTACAAGAAACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCAGCC 342 (SEQ ID NO:13)

Q V Q L Q Q P G A E L V R P G A S V K L S C K A S G Y T F T
S N Y I N W V K Q R P G Q G L E W I G N I Y P S D G F T N Y
CDR1 CDR2
N Q K F K D R A T L T V D K S S S T A Y M Q L S S P T S E D

S A V Y Y C T R N F D V W G A G T T V T V S S A (SEQ ID NO:14)
CDR3

CDR1: S N Y I N (SEQ ID NO:15)
CDR2: N I Y P S D G F T N Y N Q K F K D (SEQ ID NO:16)
CDR3: N F D V (SEQ ID NO:17)

FIG. 16A

GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGCAGATCTAGTCAGAGCCTTGTC 90
CACAGTAATGGAAACACCTATTTACAGTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTACACAGTTTCCAACCGATTT 180
TCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGCCAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTT 270
TATTTCTGCTCTCAAAGTACACATGTTCCTTTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAA 336 (SEQ ID NO:18)

D V V M T Q T P L S L P V S L G D Q A S I S C R S S Q S L V
CDR1
H S N G N T Y L Q W Y L Q K P G Q S P K L L I Y T V S N R F
CDR2
S G V P D R F S G S G S G P D F T L K I S R V E A E D L G V
Y F C S Q S T H V P F T F G S G T K L E I K (SEQ ID NO:19)
CDR3

CDR1: R S S Q S L V H S N G N T Y L Q (SEQ ID NO:20)
CDR2: T V S N R F S (SEQ ID NO:21)
CDR3: S Q S T H V P F T (SEQ ID NO:22)

FIG. 16B

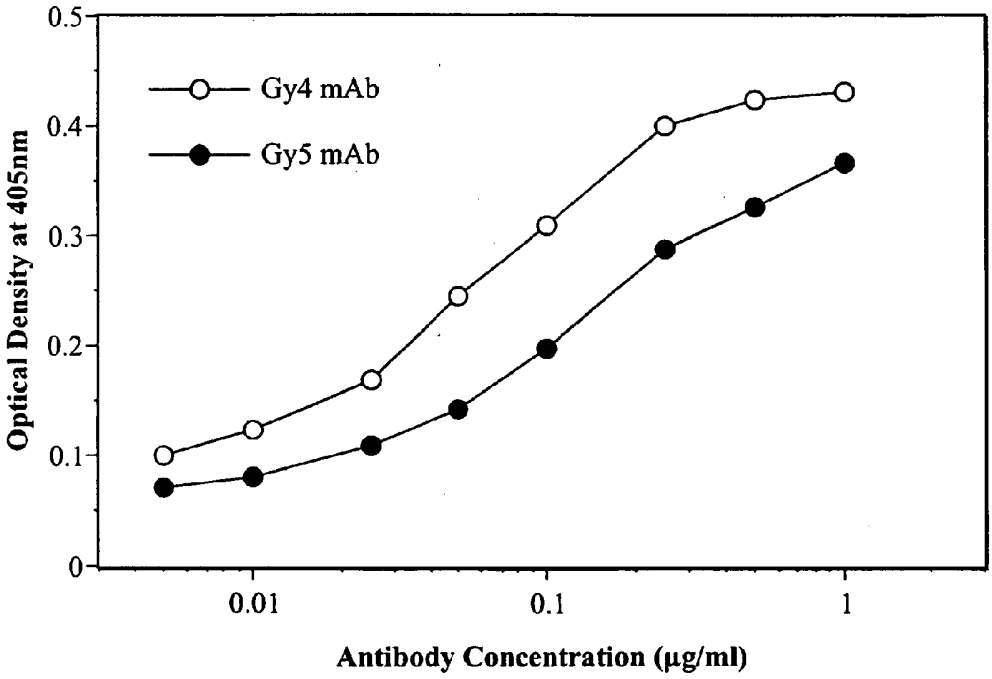

0.5
0.4
0.3
0.2
0.1
0
Optical Density at 405nm
Gy4 mAb
Gy5 mAb
0.01
0.1
1
Antibody Concentration (µg/ml)

FIG. 17

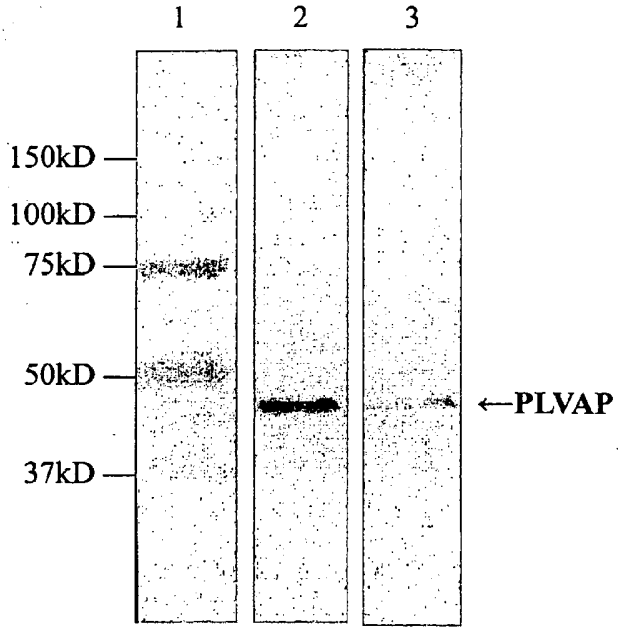

1
2
3
150kD
100kD
75kD
50kD
37kD
←PLVAP

FIG. 18

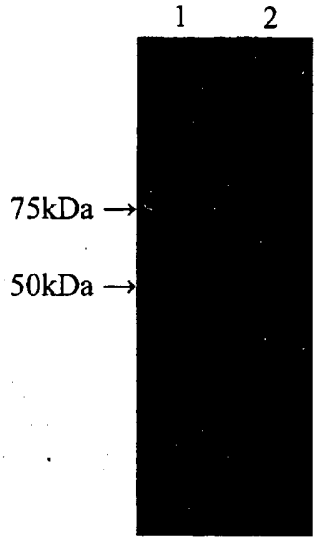


FIG. 19A

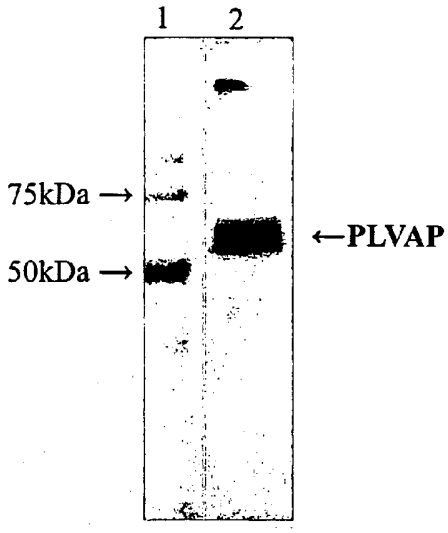


FIG. 19B

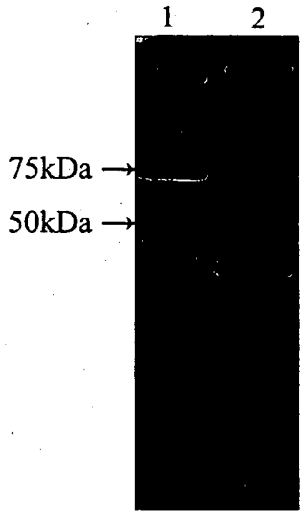


FIG. 19C

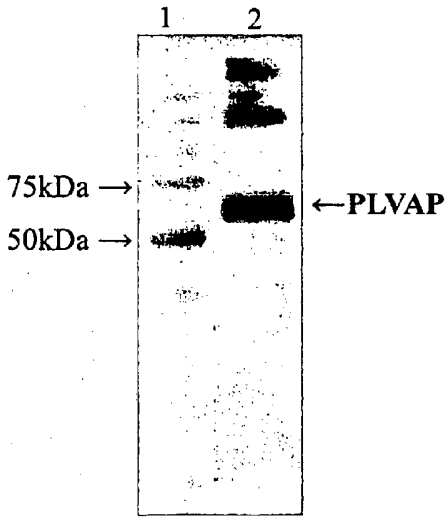


FIG. 19D

FIG. 20B

FIG. 20D

FIG. 20A

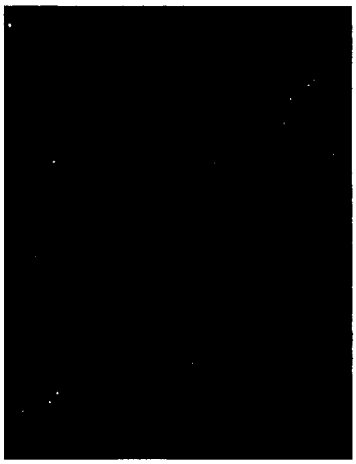


FIG. 20C

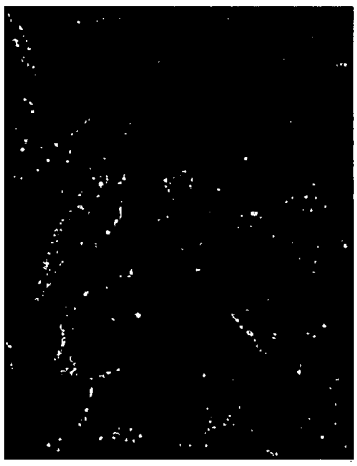


FIG. 21A

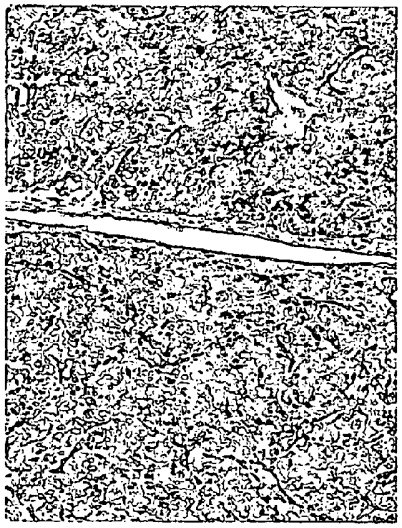


FIG. 21B

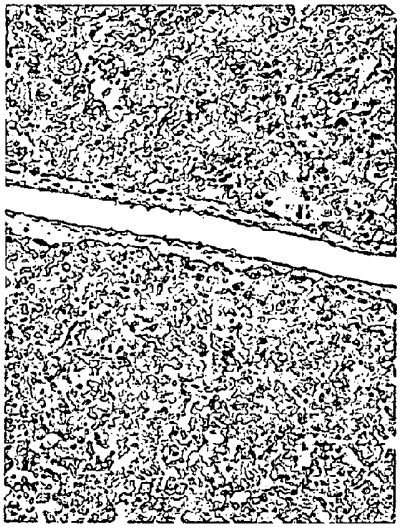


FIG. 21C

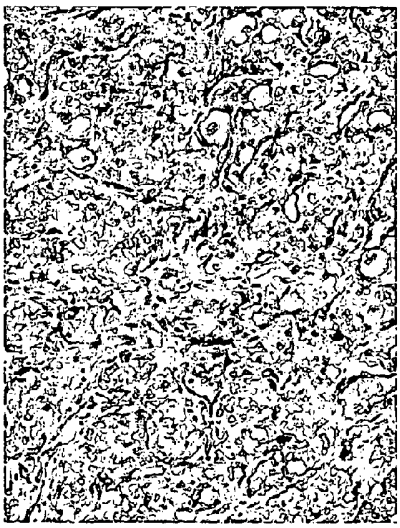
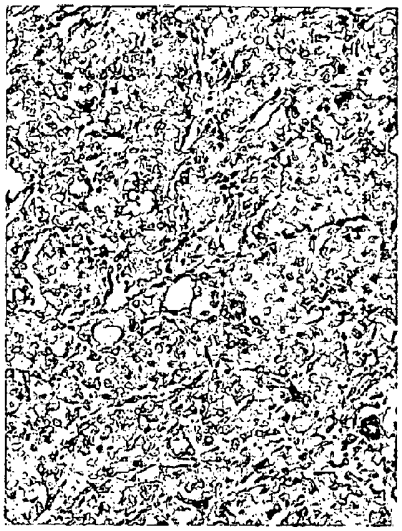


FIG. 21D



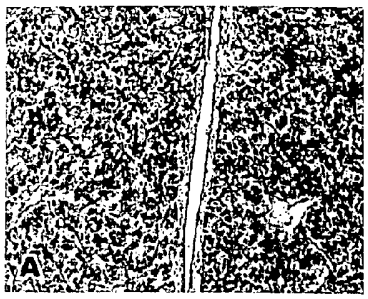


FIG. 22A

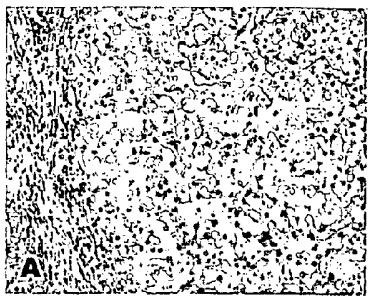


FIG. 22B

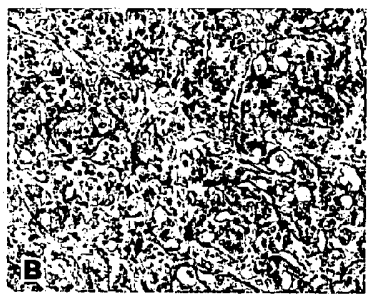


FIG. 22C

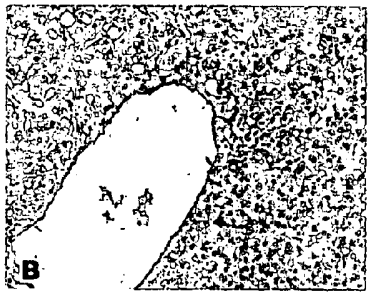


FIG. 22D

FIG. 22E

FIG. 22F

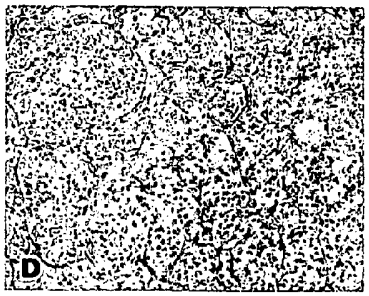


FIG. 22G

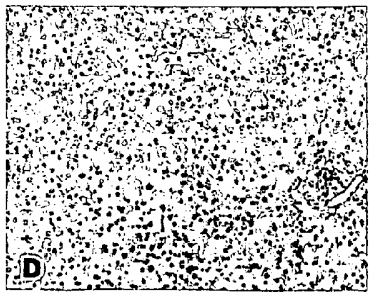


FIG. 22H

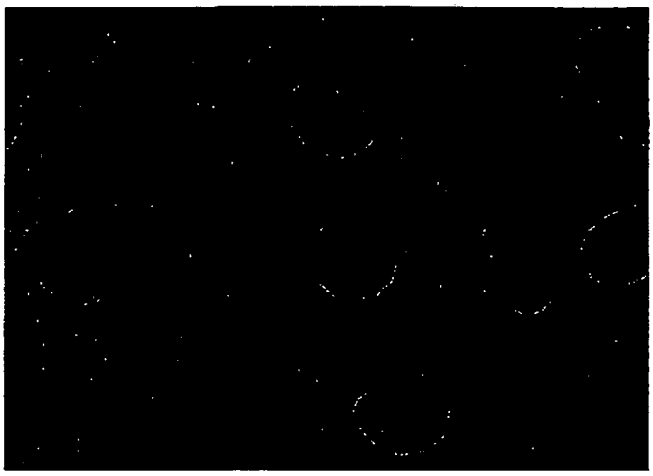


FIG. 23A

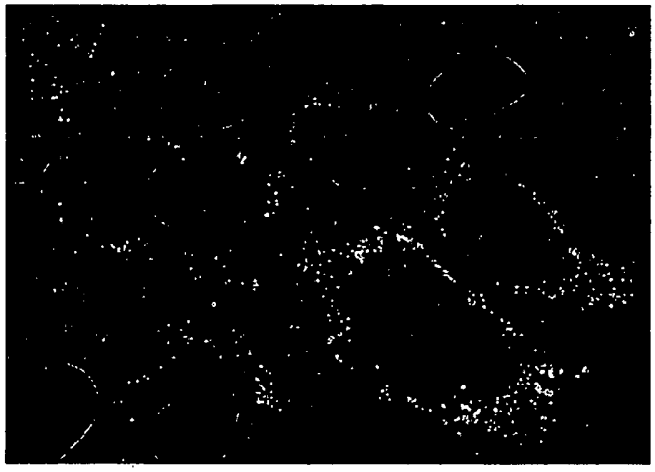


FIG. 23B

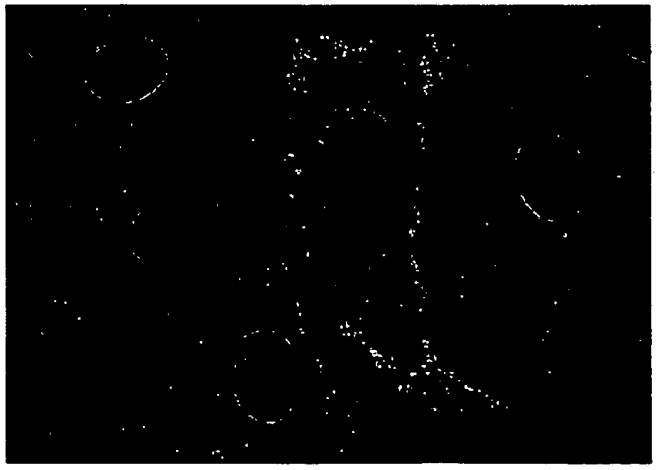


FIG. 23C

```
1   mglamehggs yaraggssrg cwyylryffl fvsliqflii lglvlfmvyg nvhvstesnl
61  qaterraegl ysqllgltas qsnltkelnf ttrakdaimq mwlnarrdld rinasfrqcq
121 gdrviytnnq rymaaiilse kqcrdqfkdm nkscdallfm lnqkvktlev eiakektict
181 kdkesvllnk rvaeeqlvec vktrelqhqe rqlakeqlqk vqalclpldk dkfemdlrnl
241 wrdsiiprsl dnlgynlyhp lgselasirr acdhmpslms skveelarsl radiervare
301 nsdlqrqkle aqqglrasqe akqkvekeaq areaklqaec srqtqlalee kavlrkerdn
361 lakeleekkr eaeqlrmela irnsaldtci ktksqpmmpv srpmgpvpnp qpidpaslee
421 fkrkilesqr ppagipvaps sg   (SEQ ID NO:23)
```

FIG. 24

```
1    cggacgcgtg ggtgagcagg gacggtgcac cggacggcgg gatcgagcaa atgggtctgg
61   ccatggagca cggagggtcc tacgctcggg cgggggggcag ctctcggggc tgctggtatt
121  acctgcgcta cttcttcctc ttcgtctccc tcatccaatt cctcatcatc ctggggctcg
181  tgctcttcat ggtctatggc aacgtgcacg tgagcacaga gtccaacctg caggccaccg
241  agcgccgagc cgagggccta tacagtcagc tcctagggct cacggcctcc cagtccaact
301  tgaccaagga gctcaacttc accacccgcg ccaaggatgc catcatgcag atgtggctga
361  atgctcgccg cgacctggac cgcatcaatg ccagcttccg ccagtgccag ggtgaccggg
421  tcatctacac gaacaatcag aggtacatgg ctgccatcat cttgagtgag aagcaatgca
481  gagatcaatt caaggacatg aacaagagct gcgatgcctt gctcttcatg ctgaatcaga
541  aggtgaagac gctggaggtg gagatagcca aggagaagac catttgcact aaggataagg
601  aaagcgtgct gctgaacaaa cgcgtggcgg aggaacagct ggttgaatgc gtgaaaaccc
661  gggagctgca gcaccaagag cgccagctgg ccaaggagca actgcaaaag gtgcaagccc
721  tctgcctgcc cctggacaag gacaagtttg agatggacct tcgtaacctg tggagggact
781  ccattatccc acgcagcctg gacaacctgg gttacaacct ctaccatccc ctgggctcgg
841  aattggcctc catccgcaga gcctgcgacc acatgcccag cctcatgagc tccaaggtgg
901  aggagctggc ccggagcctc cgggcggata tcgaacgcgt ggcccgcgag aactcagacc
961  tccaacgcca gaagctggaa gcccagcagg gcctgcgggc cagtcaggag gcgaaacaga
1021 aggtggagaa ggaggctcag gcccgggagg ccaagctcca agctgaatgc tcccggcaga
1081 cccagctagc gctggaggag aaggcggtgc tgcggaagga acgagacaac ctggccaagg
1141 agctggaaga gaagaagagg gaggcggagc agctcaggat ggagctggcc atcagaaact
1201 cagccctgga cacctgcatc aagaccaagt cgcagccgat gatgccagtg tcaaggccca
1261 tgggccctgt ccccaacccc cagcccatcg acccagctag cctggaggag ttcaagagga
1321 agatcctgga gtcccagagg ccccctgcag gcatccctgt agccccatcc agtggctgag
1381 gaggctccag gcctgaggac caagggatgg cccgactcgg cggtttgcgg aggatgcagg
1441 gatatgctca cagcgcccga cacaaccccc tcccgccgcc cccaaccacc cagggccacc
1501 atcagacaac tccctgcatg caaacccctа gtaccctctc acacccgcac ccgcgcctca
1561 cgatccctca cccagagcac acggccgcgg agatgacgtc acgcaagcaa cggcgctgac
1621 gtcacatatc accgtggtga tggcgtcacg tggccatgta gacgtcacga agagatatag
```

FIG. 25A

1681 cgatggcgtc gtgcagatgc agcacgtcgc acacagacat ggggaacttg gcatgacgtc

1741 acaccgagat gcagcaacga cgtcacgggc catgtcgacg tcacacatat taatgtcaca

1801 cagacgcggc gatggcatca cacagacggt gatgatgtca cacacagaca cagtgacaac

1861 acacaccatg acaacgacac ctatagatat ggcaccaaca tcacatgcac gcatgccctt

1921 tcacacacac tttctaccca attctcacct agtgtcacgt tcccccgacc ctggcacacg

1981 ggccaaggta cccacaggat cccatcccct cccgcacagc cctgggcccc agcacctccc

2041 ctcctccagc ttcctggcct cccagccact tcctcacccc cagtgcctgg acccggaggt

2101 gagaacagga agccattcac ctccgctcct tgagcgtgag tgtttccagg acccccctcgg

2161 ggccctgagc cgggggtgag ggtcacctgt tgtcgggagg ggagccactc cttctccccc

2221 aactcccagc cctgcctgtg gcccgttgaa atgttggtgg cacttaataa atattagtaa

2281 atccttaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaa (SEQ ID NO:24)

FIG. 25B