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(54) Title: GITR ANTIBODIES FOR THE DIAGNOSIS OF NSCLC

(57) Abstract: The present invention provides methods and compositions for detecting, diagnosing, prognosing and monitoring the progress of cancers, *e.g.*, NSCLC, of epithelial origin, *e.g.*, lung, ovarian, breast, prostate and colon cancers and malignancies and kits for use in said methods. Further provided are methods for screening to identify agonists and antagonists of antigens associated with these cancers and malignancies.

# GITR ANTIBODIES FOR THE DIAGNOSIS OF NSCLC

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. §119(e) from U.S. Provisional Patent Application Serial No. 60/645,349, filed January 19, 2005, which application is incorporated herein by reference in its entirety.

## TECHNICAL FIELD

The invention relates to compositions and methods useful in therapeutic, diagnostic and screening methods for human cancers and related malignancies.

## BACKGROUND

Despite numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Failure occurs either because the initial tumor is unresponsive, or because of recurrence due to regrowth at the original site and/or metastases.

Lung cancer is one of the most common malignancies worldwide and is the second leading cause of cancer death in man. See, American Cancer Society, Cancer facts and figures, 1996, Atlanta. Approximately 178,100 new cases of lung cancer were to be diagnosed in 1997, accounting for 13% of cancer diagnoses. An estimated 160,400 deaths due to lung cancer would occur in 1997 accounting for 29% of all cancer deaths, making lung cancer more deadly than the combination of breast, prostrate and colorectal cancers. Jemal, A. et al. (2004) Cancer Statistics 2004, CA: A Cancer Journal for Clinicians **53**:5-26. The one-year survival rates for lung cancer have increased from 32% in 1973 to 41% in 1993, largely due to improvements in

surgical techniques. The 5 year survival rate for all stages combined is only 14%. The survival rate is 48% for cases detected when the disease is still localized, but only 15% of lung cancers are discovered that early.

Among various forms of lung cancer, non-small cell lung cancer (NSCLC) accounts for nearly 80% of all new lung cancer cases each year. Small cell lung cancer is the most malignant and fastest growing form of lung cancer. The primary tumor is generally responsive to chemotherapy, but is followed by wide-spread metastasis. The median survival time at diagnosis is approximately 1 year, with a 5 year survival rate of 5%. For patients diagnosed with NSCLC, surgical resection offers the only chance of meaningful survival.

There are five types of non-small cell lung cancer: squamous cell carcinoma, adenocarcinoma, large cell carcinoma, adenosquamous carcinoma and undifferentiated carcinoma. Adenosquamous carcinomas begin in cells that appear flattened when viewed under a microscope. Undifferentiated carcinoma cells do not appear like normal cells and multiply uncontrollably. Squamous cell cancer is the most common type of lung cancer. It develops from the cells that line the airways. Adenocarcinoma develops from a glandular or secretory cells that produce mucus (phlegm). Large cell lung cancer has been thus named because the cells look large and rounded when they are viewed under a microscope.

Non-small cell cancer also is characterized by four clinical stages. Stage I is very localized cancer with no cancer in the lymph nodes. Stage II cancer has spread to the lymph nodes at the top of the affected lung. Stage III cancer has spread near to where the cancer started. This can be to the chest wall, the covering of the lung (pleura), the middle of the chest (mediastinum) or other lymph nodes. Stage IV cancer has spread to another part of the body.

Several antibody therapies are in development to treat lung cancer. Cetuximab and gifitinib are approved by the U.S. Food and Drug Administration for these cancers. Cetuximab in combination with chemotherapy has provided some benefit to NSCLC patients but further trials are still needed. Kelly, K. et al. (2003) Proc. Am. Soc. Clin. Oncol. **22**:644.

Therefore, an effective treatment for NSCLC is still required. This invention satisfies this need and provides related advantages as well.

## DISCLOSURE OF THE INVENTION

The present invention provides compositions and methods for aiding in the diagnoses of the condition of a cell, for identifying and/or distinguishing normal and neoplastic cells and for identifying potential therapeutic agents to reverse neoplasia and/or ameliorate the symptoms associated with the presence of neoplastic cells in a subject.

Accordingly, embodiments of the invention are directed to methods of diagnosing the condition of a cell by screening for the presence of a differentially expressed gene identified in Table 1. In one aspect, the differential expression of the gene is indicative of the neoplastic state of a cell of epithelial origin, *e.g.*, non-small cell lung cancer (NSCLC), ovarian, breast, prostate and colon cancers. Expression can be detected by any appropriate method, including for example, by detecting the quantity of mRNA transcribed from the gene or the quantity of cDNA produced from the reverse transcription of the mRNA transcribed from the gene or the quantity of the polypeptide or protein encoded by the gene. These methods can be performed on a sample by sample basis or modified for high throughput analysis. Additionally, databases containing quantitative full or partial transcripts or protein sequences isolated from a cell sample can be searched and analyzed for the presence and amount of transcript or expressed gene product.

Another aspect of the invention is a screen to identify therapeutic agents that reverse or treat neoplasia and tumors, wherein the cell and/or tumor is characterized by the differential expression of a polypeptide or protein identified in Table 1. The method comprises contacting the cell previously identified as possessing this genotype with an effective amount of a potential agent and assaying for reversal of the neoplastic condition.

Further provided are polynucleotides encoding the proteins, fragment(s) thereof or polypeptides shown in Table 1, (also referred to herein as gene expression product), gene delivery vehicles comprising these polynucleotides and host cells comprising these polynucleotides. The proteins, polypeptides or fragment(s) thereof

are also useful to generate antibodies that specifically recognize and bind to these molecules. The antibodies can be polyclonal or monoclonal. These antibodies can be used to isolate protein or polypeptides expressed from the genes encoding the polypeptides and to detect neoplastic cells or tumors.

The invention also provides isolated host cells and recombinant host cells that contain a polynucleotide encoding the peptides identified in Table 1 and/or fragment(s) thereof. The cells can be prokaryotic or eukaryotic and by way of example only, can be any one or more of bacterial, yeast, animal, mammalian, human and particular subtypes thereof, *e.g.*, stem cells, antigen presenting cells (APCs) such as dendritic cells (DCs) or T cells.

Table 1

| Gene | Unigene & GenBank Numbers | Locus Link ID* | Normal cell expression | Cancer cell Expression | Seq. ID Nos. |
|---|---|---|---|---|---|
| GITR (a/k/a. "TNFRSF18") | Hs.212680<br><br>AF117297.1<br>AF241229.1<br>AF125304.1<br>AY358877.1<br>NM_148901.1<br>NM_148902.1<br>NM_004195.2<br>NP_004186<br>NP_683699<br>NT_077913 | 8784 | | Adeno and squamous cancers; NSCLC; ovarian, breast; prostate and colon cancers | 1, 2 |

*web address is = ncbi.nlm.nih.gov/LocusLink/list.cgi.

Further provided by this invention is a method for monitoring a cancer in a subject by assaying, at different times, the expression level of the gene of interest and comparing the expression levels of the gene or to determine if expression has increased or decreased, thereby monitoring the cancer in the subject. A kit for use in a diagnostic method or drug screen is further provided herein. The kit comprises at least one agent (*e.g.*, probe, primer or antibody) that detects expression of the gene and instructions for use.

## BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

As used herein, the term "GITR gene" refers to at least the ORF of a contiguous polynucleotide sequence and that encodes a protein or polypeptide having the biological activity as set forth herein. LocusLink, *supra*, reports that the protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor has been reported to have increased expression upon T-cell activation, and it is thought to play a key role in dominant immunological self-tolerance maintained by CD25(+)CD4(+) regulatory T cells. Knockout studies in mice also suggest the role of this receptor is in the regulation of CD3-driven T-cell activation and programmed cell death. Three alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.

Sequence ID NO.: 1 is one example of a GITR polynucleotide sequence, and others are known in the art, examples of which include, but are not limited to the sequences set forth in Table 1, and the sequences that encode GITR gene expression products as defined herein. Also included within this definition are biologically equivalent sequences such as those sequences that code for the polypeptide of SEQ ID NO:2 and those having at least 90% or alternatively, at least 95% sequence homology to an exemplary sequence, such as SEQ ID NO.: 1, and as determined by percent identity sequence analysis run under default parameters. Also within this definition are biologically equivalent genes or polynucleotides that are identified by the ability to hybridize under conditions of high stringency to the minus strand. It may be desirable to use non-human genes, the polynucleotide sequences of which are known in the art. *See* for example, UniGene Cluster Hs.212680. Polynucleotide fragments are also known in the art, and include but are not limited to GenBank Accession numbers: BI911657.1; AI499936.1; AI214481.1; and AI923712.1. These are particularly useful as probes or primers.

As used herein, the term "GITR gene expression product, protein or polypeptide" includes the amino acid sequence of SEQ ID NO.: 2 as well as the amino acid sequences transcribed and translated from the GITR genes identified above, without regard to the gene expression system, *e.g.*, bacterial or other prokaryotic cell, yeast cell, mammalian cell such as a simian, bovine or human cell. The term includes isolated, naturally occurring polypeptides isolated from tissue

samples as well as recombinantly produced proteins and polypeptides. The term also includes polypeptides having the amino acid sequences that are at least 90 % or alternatively at least 95% homologous to SEQ ID NO.:2 and which have the biological activity as described herein. Examples of homologous amino acid sequences include, but are not limited to polypeptides have the amino acid sequence of SEQ ID NO.: 2 or other GITR gene expression product that has been modified by conservative amino acid substitutions.

## MODES FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

### Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. *See e.g.,* Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

As used herein, certain terms have the following defined meanings.

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

All numerical designations, *e.g.*, pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied ( + ) or ( - )

by increments of 0.1. It is to be understood, although not always explicitly stated that all numerical designations are preceded by the term "about". It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

The terms "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polymer. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of this invention that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

A polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for guanine when the polynucleotide is RNA. Thus, the term "polynucleotide sequence" is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

A "gene" refers to a polynucleotide containing at least one open reading frame (ORF) that is capable of encoding a particular polypeptide or protein after being transcribed and translated. Any of the polynucleotides sequences described herein

may be used to identify larger fragments or full-length coding sequences of the gene with which they are associated. Methods of isolating larger fragment sequences are known to those of skill in the art.

A "gene product" or alternatively a "gene expression product" refers to the amino acid (*e.g.*, peptide or polypeptide) generated when a gene is transcribed and translated.

The term "polypeptide" is used interchangeably with the term "protein" and in its broadest sense refers to a compound of two or more subunit amino acids, amino acid analogs or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunit may be linked by other bonds, *e.g.*, ester, ether, *etc.* As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D and L optical isomers, amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

"Under transcriptional control" is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operatively linked to an element which contributes to the initiation of, or promotes, transcription. "Operatively linked" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

The term "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody or fragment(s) thereof, are normally associated with in nature. In one aspect of this invention, an isolated polynucleotide is separated from the 3' and 5' contiguous nucleotides with which it is normally associated with in its native or natural environment, *e.g.*, on the chromosome. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody or fragment(s) thereof, does not require "isolation" to distinguish it from its naturally occurring counterpart. In addition, a "concentrated", "separated" or "diluted" polynucleotide, peptide, polypeptide, protein, antibody or fragment(s) thereof, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A polynucleotide, peptide, polypeptide, protein, antibody or fragment(s) thereof, which differs from the naturally occurring counterpart in its primary sequence or for example, by its glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally occurring counterpart by its primary sequence or, alternatively, by another characteristic such as glycosylation pattern. Thus, a non-naturally occurring polynucleotide is provided as a separate embodiment from the isolated naturally occurring polynucleotide. A protein produced in a bacterial cell is provided as a separate embodiment from the naturally occurring protein isolated from a eukaryotic cell in which it is produced in nature.

"Gene delivery," "gene transfer," and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a ""transgene") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, *e.g.*, viral infection/transfection or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the

host cell such as an extrachromosomal replicon (*e.g.*, a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known in the art to be capable of mediating transfer of genes to mammalian cells.

A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; recombinant yeast cells, metal particles; and bacteria or viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts and may be used for gene therapy as well as for simple protein expression.

A "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors, alphavirus vectors and the like. Alphavirus vectors, such as Semliki Forest virus-based vectors and Sindbis virus-based vectors, have also been developed for use in gene therapy and immunotherapy. *See,* Schlesinger and Dubensky (1999) Curr. Opin. Biotechnol. 5:434-439 and Ying *et al.* (1999) Nat. Med. 5(7):823-827. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof and a therapeutic gene. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, "retroviral vector" refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form which

integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof and a transgene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. *See e.g.*, WO 95/27071. Ads are easy to grow and do not require integration into the host cell genome. Recombinant Ad derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. *See e.g.*, WO 95/00655 and WO 95/11984. Wild-type AAV has high infectivity and specificity integrating into the host cell's genome. *See,* Hermonat and Muzyczka (1984) Proc. Natl. Acad. Sci. USA **81**:6466-6470 and Lebkowski et al. (1988) Mol. Cell. Biol. **8**:3988-3996.

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo* and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

Gene delivery vehicles also include several non-viral vectors, including DNA/liposome complexes, recombinant yeast cells and targeted viral protein-DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragment(s) thereof which bind cell surface antigens, *e.g.*, TCR, CD3 or CD4.

A "probe" when used in the context of polynucleotide manipulation refers to an oligonucleotide that is provided as a reagent to detect a target potentially present in a sample of interest by hybridizing with the target. Usually, a probe will comprise a

label or a means by which a label can be attached, either before or subsequent to the hybridization reaction. Suitable labels include, but are not limited to radioisotopes, fluorochromes, chemiluminescent compounds, dyes and proteins, including enzymes.

A "primer" is a short polynucleotide, generally with a free 3' -OH group that binds to a target or "template" potentially present in a sample of interest by hybridizing with the target and, thereafter, promoting polymerization of a polynucleotide complementary to the target. A "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using a "pair of primers" or a "set of primers" consisting of an "upstream" and a "downstream" primer and a catalyst of polymerization, such as a DNA polymerase and, typically, a thermally-stable polymerase enzyme. Methods for PCR are well-known in the art, and taught, for example in "PCR: A PRACTICAL APPROACH" (M. MacPherson *et al.*, IRL Press at Oxford University Press (1991)). All processes of producing replicate copies of a polynucleotide, such as PCR or gene cloning, are collectively referred to herein as "replication." A primer can also be used as a probe in hybridization reactions, such as Southern or Northern blot analyses. Sambrook *et al.*, *supra*.

An expression "database" denotes a set of stored data that represent a collection of sequences, which in turn represent a collection of biological reference materials.

The term "cDNAs" refers to complementary DNA that is mRNA molecules present in a cell or organism made into cDNA with an enzyme such as reverse transcriptase. A "cDNA library" is a collection of all of the mRNA molecules present in a cell or organism, all turned into cDNA molecules with the enzyme reverse transcriptase, then inserted into "vectors" (other DNA molecules that can continue to replicate after addition of foreign DNA). Exemplary vectors for libraries include bacteriophage (also known as "phage"), viruses that infect bacteria, for example, lambda phage. The library can then be probed for the specific cDNA (and thus mRNA) of interest.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides or proteins. If the

polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell. "Differentially expressed" as applied to a gene, refers to the differential production of the mRNA transcribed and/or translated from the gene or the protein product encoded by the gene. A differentially expressed gene may be overexpressed or underexpressed as compared to the expression level of a normal or control cell. However, as used herein overpression as at least 1.25 fold or, alternatively, at least 1.5 fold or, alternatively, at least 2 fold expression over that detected in a normal or healthy counterpart cell or tissue. The term "differentially expressed" also refers to nucleotide sequences in a cell or tissue which are expressed where silent in a control cell or not expressed where expressed in a control cell.

As used herein, "solid phase support" or "solid support", used interchangeably, is not limited to a specific type of support. Rather a large number of supports are available and are known to one of ordinary skill in the art. Solid phase supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, alumina gels, microarrays and chips. As used herein, "solid support" also includes synthetic antigen-presenting matrices, cells and liposomes. A suitable solid phase support may be selected on the basis of desired end use and suitability for various protocols. For example, for peptide synthesis, solid phase support may refer to resins such as polystyrene (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, *etc.*), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Biosearch, California).

A polynucleotide also can be attached to a solid support for use in high throughput screening assays. PCT WO 97/10365, for example, discloses the construction of high density oligonucleotide chips. *See also*, U.S. Patent Nos. 5,405,783; 5,412,087; and 5,445,934. Using this method, the probes are synthesized on a derivatized glass surface also known as chip arrays. Photoprotected nucleoside phosphoramidites are coupled to the glass surface, selectively deprotected by photolysis through a photolithographic mask and reacted with a second protected nucleoside phosphoramidite. The coupling/deprotection process is repeated until the desired probe is complete.

"Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction or the enzymatic cleavage of a polynucleotide by a ribozyme.

Hybridization reactions can be performed under conditions of different "stringency". In general, a low stringency hybridization reaction is carried out at about 40 $^0$C in 10x SSC or a solution of equivalent ionic strength/temperature. A moderate stringency hybridization is typically performed at about 50 $^0$C in 6x SSC, and a high stringency hybridization reaction is generally performed at about 60 $^0$C in 1x SSC.

When hybridization occurs in an antiparallel configuration between two single-stranded polynucleotides, the reaction is called "annealing" and those polynucleotides are described as "complementary". A double-stranded polynucleotide can be "complementary" or "homologous" to another polynucleotide, if hybridization can occur between one of the strands of the first polynucleotide and the second. "Complementarity" or "homology" (the degree that one polynucleotide is complementary with another) is quantifiable in terms of the proportion of bases in opposing strands that are expected to form hydrogen bonding with each other, according to generally accepted base-pairing rules.

A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 80%, 85%, 90% or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F.M. Ausubel *et al.*, eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. Preferably, default parameters are used for alignment. A

preferred alignment program is BLAST, using default parameters. In particular, preferred programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: www.ncbi.nlm.nih.gov/cgi-bin/BLAST.

Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. Metaplasia is a form of controlled cell growth in which one type of fully differentiated cell substitutes for another type of differentiated cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (*i.e.*, de-regulated cell division). Neoplastic cells can be malignant or benign. A metastatic cell or tissue means that the cell can invade and destroy neighboring body structures.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without therapeutic intervention. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a $^{3}$H-thymidine incorporation assay or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying and stopping tumor growth, as well as tumor shrinkage.

The term "antigen" is well understood in the art and includes substances which are immunogenic. The term as used herein also includes substances which induce immunological unresponsiveness or anergy.

A "native" or "natural" or "wild-type" antigen is a polypeptide, protein or a fragment which contains an epitope and which has been isolated from a natural biological source. It also can specifically bind to an antigen receptor.

As used herein, an "antibody" includes whole antibodies and any antigen binding fragment or a single chain thereof. Thus the term "antibody" includes any protein or peptide containing molecule that comprises at least a portion of an immunoglobulin molecule. Examples of such include, but are not limited to a complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework (FR) region, or any portion thereof, or at least one portion of a binding protein, any of which can be incorporated into an antibody of the present invention.

The antibodies can be polyclonal or monoclonal and can be isolated from any suitable biological source, *e.g.*, murine, rat, sheep and canine. Additional sources are identified *infra*.

The term "antibody" is further intended to encompass digestion fragments, specified portions, derivatives and variants thereof, including antibody mimetics or comprising portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof. Examples of binding fragments encompassed within the term "antigen binding portion" of an antibody include a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH, domains; a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the VH and CH, domains; a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, a dAb fragment (Ward et al. (1989) Nature **341**:544-546), which consists of a VH domain; and an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv)). Bird et al. (1988) Science **242**:423-426 and Huston et al. (1988) Proc. Natl. Acad Sci. USA **85**:5879-5883. Single chain

antibodies are also intended to be encompassed within the term "fragment of an antibody." Any of the above-noted antibody fragments are obtained using conventional techniques known to those of skill in the art, and the fragments are screened for binding specificity and neutralization activity in the same manner as are intact antibodies.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The term "antibody derivative" is intended to encompass molecules that bind an epitope as defined above and which are modifications or derivatives of a native monoclonal antibody of this invention. Derivatives include, but are not limited to, for example, bispecific, multispecific, heterospecific, trispecific, tetraspecific, multispecific antibodies, diabodies, chimeric, recombinant and humanized.

The term "bispecific molecule" is intended to include any agent, *e.g.*, a protein, peptide, or protein or peptide complex, which has two different binding specificities. The term "multispecific molecule" or "heterospecific molecule" is intended to include any agent, *e.g.* a protein, peptide, or protein or peptide complex, which has more than two different binding specificities.

The term "heteroantibodies" refers to two or more antibodies, antibody binding fragments (*e.g.*, Fab), derivatives thereof, or antigen binding regions linked together, at least two of which have different specificities.

The term "human antibody" as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody" as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian

species, such as a mouse, have been grafted onto human framework sequences. Thus, as used herein, the term "human antibody" refers to an antibody in which substantially every part of the protein (*e.g.*, CDR, framework, $C_L$, $C_H$ domains (*e.g.*, $C_{H1}$, $C_{H2}$, $C_{H3}$), hinge, ($V_L$, $V_H$)) is substantially non-immunogenic in humans, with only minor sequence changes or variations. Similarly, antibodies designated primate (monkey, baboon, chimpanzee, etc.), rodent (mouse, rat, rabbit, guinea pig, hamster, and the like) and other mammals designate such species, sub-genus, genus, sub-family, family specific antibodies. Further, chimeric antibodies include any combination of the above. Such changes or variations optionally and preferably retain or reduce the immunogenicity in humans or other species relative to non-modified antibodies. Thus, a human antibody is distinct from a chimeric or humanized antibody. It is pointed out that a human antibody can be produced by a non-human animal or prokaryotic or eukaryotic cell that is capable of expressing functionally rearranged human immunoglobulin (*e.g.*, heavy chain and/or light chain) genes. Further, when a human antibody is a single chain antibody, it can comprise a linker peptide that is not found in native human antibodies. For example, an Fv can comprise a linker peptide, such as two to about eight glycine or other amino acid residues, which connects the variable region of the heavy chain and the variable region of the light chain. Such linker peptides are considered to be of human origin.

As used herein, a human antibody is "derived from" a particular germline sequence if the antibody is obtained from a system using human immunoglobulin sequences, *e.g.*, by immunizing a transgenic mouse carrying human immunoglobulin genes or by screening a human immunoglobulin gene library. A human antibody that is "derived from" a human germline immunoglobulin sequence can be identified as such by comparing the amino acid sequence of the human antibody to the amino acid sequence of human germline immunoglobulins. A selected human antibody typically is at least 90% identical in amino acids sequence to an amino acid sequence encoded by a human germline immunoglobulin gene and contains amino acid residues that identify the human antibody as being human when compared to the germline immunoglobulin amino acid sequences of other species (*e.g.*, murine germline sequences). In certain cases, a human antibody may be at least 95%, or even at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene. Typically, a human antibody derived

from a particular human germline sequence will display no more than 10 amino acid differences from the amino acid sequence encoded by the human germline immunoglobulin gene. In certain cases, the human antibody may display no more than 5, or even no more than 4, 3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

A "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences.

The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom, antibodies isolated from a host cell transformed to express the antibody, *e.g.*, from a transfectoma, antibodies isolated from a recombinant, combinatorial human antibody library, and antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgG1) that is encoded by heavy chain constant region genes.

The terms "transgenic, nonhuman animal" refers to a nonhuman animal having a genome comprising one or more human heavy and/or light chain transgenes or transchromosomes (either integrated or non-integrated into the animal's natural genomic DNA) and which is capable of expressing fully human antibodies. For example, a transgenic rat can have a human light chain transgene and either a human heavy chain transgene or human heavy chain transchromosome, such that the rat produces human anti-INF-$\alpha$ antibodies. The human heavy chain transgene can be integrated into the chromosomal DNA of the rat, or the human heavy chain transgene can be maintained extrachromosomally. Transgenic and transchromosomal animals are capable of producing multiple isotypes of human monoclonal antibodies to Alpha V (*e.g.*, IgG, IgA and/or IgE) by undergoing V-D-J recombination and isotype switching.

A "composition" is also intended to encompass a combination of active agent and another carrier, *e.g.*, compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant, diluent, binder, stabilizer, buffers, salts, lipophilic solvents, preservative, adjuvant or the like. Carriers also include pharmaceutical excipients and additives proteins, peptides, amino acids, lipids, and carbohydrates (*e.g.*, sugars, including monosaccharides, di-, tri-, tetra-, and oligosaccharides; derivatized sugars such as alditols, aldonic acids, esterified sugars and the like; and polysaccharides or sugar polymers), which can be present singly or in combination, comprising alone or in combination 1-99.99% by weight or volume. Exemplary protein excipients include serum albumin such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, and the like. Representative amino acid/antibody components, which can also function in a buffering capacity, include alanine, glycine, arginine, betaine, histidine, glutamic acid, aspartic acid, cysteine, lysine, leucine, isoleucine, valine, methionine, phenylalanine, aspartame, and the like. Carbohydrate excipients are also intended within the scope of this invention, examples of which include but are not limited to monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol) and myoinositol.

The term carrier further includes a buffer or a pH adjusting agent; typically, the buffer is a salt prepared from an organic acid or base. Representative buffers include organic acid salts such as salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid, acetic acid, or phthalic acid; Tris, tromethamine hydrochloride, or phosphate buffers. Additional carriers include polymeric excipients/additives such as polyvinylpyrrolidones, ficolls (a polymeric sugar), dextrates (*e.g.*, cyclodextrins, such as 2-hydroxypropyl-.quadrature.-cyclodextrin), polyethylene glycols, flavoring agents, antimicrobial agents, sweeteners, antioxidants, antistatic agents, surfactants (*e.g.*, polysorbates such as "TWEEN 20" and "TWEEN 80"), lipids (*e.g.*, phospholipids, fatty acids), steroids (*e.g.*, cholesterol), and chelating agents (*e.g.*, EDTA).

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives and any of the above noted carriers with the additional provisio that they be acceptable for use *in vivo*. For examples of carriers, stabilizers and adjuvants, see Martin REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975) and Williams & Williams, (1995), and in the "PHYSICIAN'S DESK REFERENCE", 52nd ed., Medical Economics, Montvale, N.J. (1998).

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

The invention provides an antibody or a variant, derivative or fragment thereof that specifically recognizes and binds an epitope on the polgreplide or protein expressed by a gene identified in Table 1. In one aspect, the antibodies are isolated. In another aspect, they are combined with a suitable carrier. The antibodies can be polyclonal or monoclonal and can be isolated from any species, murine, rat, simian, or recobminantly produced and isolated. Also provided by this invention are the hybridoma cell lines that produce these monoclonal antibodies, alone in combination with a carrier or in culture.

Also provided by this invention is a polypeptide that comprises an antibody, variant, derivative or fragment thereof, including but not limited to immunoglobulin chains and CDRs.

The present invention further provides an anti-idiotypic antibody. An anti-idiotype antibody includes any protein or peptide containing molecule that comprises at least a portion of an immunoglobulin molecule, such as but not limited to at least one complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework region, or any portion thereof, that can be incorporated into an antibody of the present invention. An anti-idiotype antibody of the invention can include or be derived from any mammal, such as but not limited to a human, a mouse, a rabbit, a rat, a rodent, a primate, and the like.

This invention further provides an isolated polynucleotide that encodes an antibody of this invention, alone in combination with vectors, carriers, pharmaceutically acceptable carriers, diluents, and host cells.

The present invention further provides, in one aspect, isolated nucleic acid molecules comprising, complementary, or hybridizing to, a polynucleotide encoding at least one antibody or anti-idiotype antibody of this invention, comprising at least one specified sequence, domain, portion or variant thereof. The present invention further provides recombinant vectors comprising the nucleotides, host cells containing the nucleic acids and/or recombinant vectors, as well as methods of making and/or using such nucleic acids, vectors and/or host cells. Methods for isolating, replicating and expressing polynucleotides are known in the art and described *infra*.

One or more of the above can be further combined with a carrier, a pharmaceutically acceptable carrier or medical device which is suitable for use of the antibody or related composition in diagnostic or therapeutic methods.

The carrier can be a liquid phase carrier or solid phase carrier, *e.g.*, bead, gel or carrier molecule such as a liposome. The composition can optionally further comprise at least one further compound, protein or composition.

An additional example of "carriers" includes therapeutically active agents such as another peptide or protein (*e.g.*, an Fab' fragment). For example, an antibody

of this invention, variant, derivative or fragment thereof can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (*e.g.*, to produce a bispecific or a multispecific antibody), a cytotoxin, a cellular ligand or an antigen. Accordingly, this invention encompasses a large variety of antibody conjugates, bi- and multispecific molecules, and fusion proteins, whether or not they target the same epitope as the antibodies of this invention.

Yet additional examples of carriers are organic molecules (also termed modifying agents) or activating agents, that can be covalently attached, directly or indirectly, to an antibody of this invention. Attachment of the molecule can improve pharmacokinetic properties (*e.g.*, increased *in vivo* serum half-life). Examples of organic molecules include, but are not limited to a hydrophilic polymeric group, a fatty acid group or a fatty acid ester group. As used herein, the term "fatty acid" encompasses mono-carboxylic acids and di-carboxylic acids. A "hydrophilic polymeric group," as the term is used herein, refers to an organic polymer that is more soluble in water than in octane.

Hydrophilic polymers suitable for modifying antibodies of the invention can be linear or branched and include, for example, polyalkane glycols (*e.g.*, PEG, monomethoxy-polyethylene glycol (mPEG), PPG and the like), carbohydrates (*e.g.*, dextran, cellulose, oligosaccharides, polysaccharides and the like), polymers of hydrophilic amino acids (*e.g.*, polylysine, polyarginine, polyaspartate and the like), polyalkane oxides (*e.g.*, polyethylene oxide, polypropylene oxide and the like) and polyvinyl pyrolidone. A suitable hydrophilic polymer that modifies the antibody of the invention has a molecular weight of about 800 to about 150,000 Daltons as a separate molecular entity. The hydrophilic polymeric group can be substituted with one to about six alkyl, fatty acid or fatty acid ester groups. Hydrophilic polymers that are substituted with a fatty acid or fatty acid ester group can be prepared by employing suitable methods. For example, a polymer comprising an amine group can be coupled to a carboxylate of the fatty acid or fatty acid ester, and an activated carboxylate (*e.g.*, activated with N, N-carbonyl diimidazole) on a fatty acid or fatty acid ester can be coupled to a hydroxyl group on a polymer.

Fatty acids and fatty acid esters suitable for modifying antibodies of the invention can be saturated or can contain one or more units of unsaturation. Examples of such include, but are not limited to n-dodecanoate, n-tetradecanoate, n-octadecanoate, n-eicosanoate, n-docosanoate, n-triacontanoate, n-tetracontanoate, cis-.DELTA.9-octadecanoate, all cis-.DELTA.5,8,11,14-eicosatetraenoate, octanedioic acid, tetradecanedioic acid, octadecanedioic acid, docosanedioic acid, and the like. Suitable fatty acid esters include mono-esters of dicarboxylic acids that comprise a linear or branched lower alkyl group. The lower alkyl group can comprise from one to about twelve, preferably one to about six, carbon atoms.

In yet another aspect, the present invention provides a transgenic nonhuman animal, such as a transgenic mouse (also referred to herein as a "HuMAb mouse"), which expresses a fully human monoclonal antibody that neutralizes at least one protein subtype similar to an antibody of this invention as defined above. In a particular embodiment, the transgenic nonhuman animal is a transgenic mouse having a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an anti-alpha V antibody of the invention. Preferably, the transgenic nonhuman animal, *e.g.*, the transgenic mouse, is capable of producing multiple isotypes of human monoclonal antibodies to an epitope of interest by undergoing V-D-J recombination and isotype switching. Isotype switching may occur by, *e.g.*, classical or non-classical isotype switching.

Accordingly, in another embodiment, the invention provides isolated cells derived or isolated from a transgenic nonhuman animal as described above, *e.g.*, a transgenic mouse, which express human antibodies. The isolated B-cells can then be immortalized by fusion to an immortalized cell to provide a source (*e.g.*, a hybridoma) of human antibodies. These hybridomas are also included within the scope of the invention.

The present invention further provides at least one antibody method or composition, for diagnosing a cancer of epithelial origin, *e.g.*, non-small cell lung cancer (NSCLC), ovarian, breast, prostate or colon cancer, in a cell, tissue, organ, animal or patient and/or, prior to, subsequent to, or during a related condition, as known in the art and/or as described herein. They are also used to prognose or monitor disease progression.

Also provided is a composition containing at least one antibody of this invention, variant, derivative or fragment thereof, suitable for administration in an effective amount to modulate or ameliorate symptoms associated with cancers of epithelial origin, *e.g.*, non-small cell lung cancer (NSCLC), ovarian, breast, prostate and colon cancer, or treat at least one such cancer. The compositions include, for example, pharmaceutical and diagnostic compositions/kits, comprising a pharmaceutically acceptable carrier and at least one antibody of this invention, variant, derivative or fragment thereof. As noted above, the composition can further comprise additional antibodies or therapeutic agents which in combination, provide multiple therapies tailored to provide the maximum therapeutic benefit.

Alternatively, a composition of this invention can be co-administered with other therapeutic and cytotoxic agents, whether or not linked to them or administered in the same dosing. They can be coadministered simultaneously with such agents (*e.g.*, in a single composition or separately) or can be administered before or after administration of such agents. Such agents can include corticosteroids, nonsteroidal immune suppressants, antimalarials, and nonsteroidal anti-inflammatory drugs. The compositions can be combined with alternative therapies such as administration of corticosteroids, nonsteroidal immune suppressants, antimalarials, and nonsteroidal anti-inflamatory drugs.

The methods of this invention can be practiced either *in vitro* or *in vivo*. When practiced *in vitro*, the methods require contacting the cells with (*e.g.*, administering or delivering to the cells) one or more antibodies and/or related therapeutic compositions, derivatives etc. containing the antibodies as described above.

The antibodies and compositions can be delivered by any suitable means and with any suitable formulation. Accordingly, a formulation comprising an antibody of this invention is further provided herein. The formulation can further comprise one or more preservative or stabilizer such as phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, phenylmercuric nitrite, phenoxyethanol, formaldehyde, chlorobutanol, magnesium chloride (*e.g.*, hexahydrate), alkylparaben (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof in an aqueous diluent. Any suitable concentration or mixture can be used as known in the art, such as 0.001-5%,

or any range or value therein, such as, but not limited to 0.001, 0.003, 0.005, 0.009, 0.01, 0.02, 0.03, 0.05, 0.09, 0.1, 0.2, 0.3, 0.4., 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.3, 4.5, 4.6, 4.7, 4.8, 4.9, or any range or value therein. Non-limiting examples include, no preservative, 0.1 -2% m-cresol (*e.g.*, 0.2, 0.3. 0.4, 0.5, 0.9, 1.0%), 0.1-3% benzyl alcohol (*e.g.*, 0.5, 0.9, 1.1., 1.5, 1.9, 2.0, 2.5%), 0.001-0.5% thimerosal (*e.g.*, 0.005, 0.01), 0.001-2.0% phenol (*e.g.*, 0.05, 0.25, 0.28, 0.5, 0.9, 1.0%), 0.0005-1.0% alkylparaben(s) (*e.g.*, 0.00075, 0.0009, 0.001, 0.002, 0.005, 0.0075, 0.009, 0.01, 0.02, 0.05, 0.075, 0.09, 0.1 , 0.2, 0.3, 0.5, 0.75, 0.9, and 1.0%).

As noted above, the invention provides an article of manufacture, comprising packaging material and at least one vial comprising a solution of at least antibody as of this invention with the prescribed buffers and/or preservatives, optionally in an aqueous diluent, wherein said packaging material comprises a label that indicates that such solution can be held over a period of 1, 2, 3, 4, 5, 6, 9, 12, 18, 20, 24, 30, 36,40, 48, 54, 60, 66, 72 hours or greater. The invention further comprises an article of manufacture, comprising packaging material, a first vial comprising at least one lyophilized antibody of this invention and a second vial comprising an aqueous diluent of prescribed buffer or preservative, wherein said packaging material comprises a label that instructs a patient to reconstitute the antibody in the aqueous diluent to form a solution that can be held over a period of twenty-four hours or greater.

The range antibody includes amounts yielding upon reconstitution, if in a wet/dry system, concentrations from about 1.0 $\mu$g/ml to about 1000 mg/ml, although lower and higher concentrations are operable and are dependent on the intended delivery vehicle, *e.g.*, solution formulations will differ from transdermal patch, pulmonary, transmucosal, or osmotic or micro pump methods.

The formulations of the present invention can be prepared by a process which comprises mixing at least one antibody of this invention and a preservative selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, alkylparaben, (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal or mixtures

thereof in an aqueous diluent. Mixing of the antibody and preservative in an aqueous diluent is carried out using conventional dissolution and mixing procedures. For example, a measured amount of at least one antibody in buffered solution is combined with the desired preservative in a buffered solution in quantities sufficient to provide the antibody and preservative at the desired concentrations. Variations of this process would be recognized by one of ordinary skill in the art, *e.g.*, the order the components are added, whether additional additives are used, the temperature and pH at which the formulation is prepared, are all factors that can be optimized for the concentration and means of administration used.

The compositions and formulations can be provided to patients as clear solutions or as dual vials comprising a vial of lyophilized antibody that is reconstituted with a second vial containing the aqueous diluent. Either a single solution vial or dual vial requiring reconstitution can be reused multiple times and can suffice for a single or multiple cycles of patient treatment and thus provides a more convenient treatment regimen than currently available. Recognized devices comprising these single vial systems include those pen-injector devices for delivery of a solution such as BD Pens, BD Autojectore, Humaject.RTM.' NovoPen.RTM., B-D.RTM.Pen, AutoPen.RTM., and OptiPen.RTM., GenotropinPen.RTM., Genotronorm Pen.RTM., Humatro Pen.RTM., Reco-Pen.RTM., Roferon Pen.RTM., Biojector.RTM., iject.RTM., J-tip Needle-Free Injector.RTM., Intraject.RTM., Medi-Ject.RTM., *e.g.*, as made or developed by Becton Dickensen (Franklin Lakes, N.J. available at bectondickenson.com), Disetronic (Burgdorf, Switzerland, available at disetronic.com; Bioject, Portland, Oregon (available at bioject.com); National Medical Products, Weston Medical (Peterborough, UK, available at weston-medical.com), Medi-Ject Corp (Minneapolis, Minn., available at mediject.com).

**ANTIBODIES**

The antibodies of this invention are monoclonal antibodies, although in certain aspects, polyclonal antibodies can be utilized. They also can be functional fragments, antibody derivatives or antibody variants. They can be chimeric, humanized, or totally human. A functional fragment of an antibody includes but is not limited to Fab, Fab', Fab2, Fab'2, and single chain variable regions. Antibodies can be produced in cell culture, in phage, or in various animals, including but not limited to

cows, rabbits, goats, mice, rats, hamsters, guinea pigs, sheep, dogs, cats, monkeys, chimpanzees, apes, etc. So long as the fragment or derivative retains specificity of binding or neutralization ability as the antibodies of this invention it can be used. Antibodies can be tested for specificity of binding by comparing binding to appropriate antigen to binding to irrelevant antigen or antigen mixture under a given set of conditions. If the antibody binds to the appropriate antigen at least 2, 5, 7, and preferably 10 times more than to irrelevant antigen or antigen mixture then it is considered to be specific. Specific assays, *e.g.*, ELISA, for determining specificity are known in the art.

The antibodies also are characterized by their ability to specifically recognize and bind an epitope of interest.

The monoclonal antibodies of the invention can be generated using conventional hybridoma techniques known in the art and well-described in the literature. For example, a hybridoma is produced by fusing a suitable immortal cell line (*e.g.*, a myeloma cell line such as, but not limited to, Sp2/0, Sp2/0-AG14, NSO, NS1, NS2, AE-1, L.5, >243, P3X63Ag8.653, Sp2 SA3, Sp2 MAI, Sp2 SS1, Sp2 SA5, U397, MLA 144, ACT IV, MOLT4, DA-1, JURKAT, WEHI, K-562, COS, RAJI, NIH 3T3, HL-60, MLA 144, NAMAIWA, NEURO 2A, CHO, PerC.6, YB2/O) or the like, or heteromyelomas, fusion products thereof, or any cell or fusion cell derived therefrom, or any other suitable cell line as known in the art (see, *e.g.*, www.atcc.org, www.lifetech.com., and the like), with antibody producing cells, such as, but not limited to, isolated or cloned spleen, peripheral blood, lymph, tonsil, or other immune or B cell containing cells, or any other cells expressing heavy or light chain constant or variable or framework or CDR sequences, either as endogenous or heterologous nucleic acid, as recombinant or endogenous, viral, bacterial, algal, prokaryotic, amphibian, insect, reptilian, fish, mammalian, rodent, equine, ovine, goat, sheep, primate, eukaryotic, genomic DNA, cDNA, rDNA, mitochondrial DNA or RNA, chloroplast DNA or RNA, hnRNA, mRNA, tRNA, single, double or triple stranded, hybridized, and the like or any combination thereof. Antibody producing cells can also be obtained from the peripheral blood or, preferably the spleen or lymph nodes, of humans or other suitable animals that have been immunized with the antigen of interest. Any other suitable host cell can also be used for expressing-heterologous or

endogenous nucleic acid encoding an antibody, specified fragment or variant thereof, of the present invention. The fused cells (hybridomas) or recombinant cells can be isolated using selective culture conditions or other suitable known methods, and cloned by limiting dilution or cell sorting, or other known methods.

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, but not limited to, methods that select recombinant antibody from a peptide or protein library (*e.g.*, but not limited to, a bacteriophage, ribosome, oligonucleotide, RNA, cDNA, or the like, display library; *e.g.*, as available from various commercial vendors such as Cambridge Antibody Technologies (Cambridgeshire, UK), MorphoSys (Martinsreid/Planegg, Del.), Biovation (Aberdeen, Scotland, UK) BioInvent (Lund, Sweden), using methods known in the art. See U.S. Pat. Nos. 4,704,692; 5,723,323; 5,763,192; 5,814,476; 5,817,483; 5,824,514; 5,976,862. Alternative methods rely upon immunization of transgenic animals (*e.g.*, SCID mice, Nguyen et al. (1977) Microbiol. Immunol. **41**:901-907 (1997); Sandhu et al., (1996) Crit. Rev. Biotechnol. **16**:95-118; Eren et al. (1998) Immunol. **93**:154-161 that are capable of producing a repertoire of human antibodies, as known in the art and/or as described herein. Such techniques, include, but are not limited to, ribosome display (Hanes et al. (1997) Proc. Natl. Acad. Sci. USA, **94**:4937-4942; Hanes et al., (1998) Proc. Natl. Acad. Sci. USA, **95**:14130-14135); single cell antibody producing technologies (*e.g.*, selected lymphocyte antibody method ("SLAM") (U.S. Pat. No. 5,627,052, Wen et al. (1987) J. Immunol. **17**:887-892; Babcook et al., Proc. Natl. Acad. Sci. USA (1996) **93**:7843-7848); gel microdroplet and flow cytometry (Powell et al. (1990) Biotechnol. **8**:333-337; One Cell Systems, (Cambridge, Mass).; Gray et al. (1995) J. Imm. Meth. **182**:155-163; Kenny et al. (1995) Bio/Technol. **13**:787-790); B-cell selection (Steenbakkers et al. (1994) Molec. Biol. Reports **19**:125-134 (1994).

Antibody variants of the present invention can also be prepared using delivering a polynucleotide encoding an antibody of this invention to a suitable host such as to provide transgenic animals or mammals, such as goats, cows, horses, sheep, and the like, that produce such antibodies in their milk. These methods are known in the art and are described for example in U.S. Pat. Nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; and 5,304,489.

The term "antibody variant" includes post-translational modification to linear polypeptide sequence of the antibody or fragment. For example, U.S. Patent No. 6,602,684 B1 describes a method for the generation of modified glycol-forms of antibodies, including whole antibody molecules, antibody fragments, or fusion proteins that include a region equivalent to the Fc region of an immunoglobulin, having enhanced Fc-mediated cellular toxicity, and glycoproteins so generated.

Antibody variants also can be prepared by delivering a polynucleotide of this invention to provide transgenic plants and cultured plant cells (*e.g.*, but not limited to tobacco, maize, and duckweed) that produce such antibodies, specified portions or variants in the plant parts or in cells cultured therefrom. For example, Cramer et al. (1999) Curr. Top. Microbol. Immunol. **240**:95-118 and references cited therein, describe the production of transgenic tobacco leaves expressing large amounts of recombinant proteins, *e.g.*, using an inducible promoter. Transgenic maize have been used to express mammalian proteins at commercial production levels, with biological activities equivalent to those produced in other recombinant systems or purified from natural sources. See, *e.g.*, Hood et al., Adv. Exp. Med. Biol. (1999) **464**:127-147 and references cited therein. Antibody variants have also been produced in large amounts from transgenic plant seeds including antibody fragments, such as single chain antibodies (scFv's), including tobacco seeds and potato tubers. See, *e.g.*, Conrad et al.(1998) Plant Mol. Biol. **38**:101-109 and reference cited therein. Thus, antibodies of the present invention can also be produced using transgenic plants, according to know methods.

Antibody derivatives can be produced, for example, by adding exogenous sequences to modify immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic. Generally part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the variable and constant regions are replaced with human or other amino acids.

In general, the CDR residues are directly and most substantially involved in influencing antigen binding. Humanization or engineering of antibodies of the present invention can be performed using any known method, such as but not limited to those described in U.S. Pat. Nos. 5,723,323, 5,976,862, 5,824,514, 5,817,483,

5,814,476, 5,763,192, 5,723,323, 5,766,886, 5,714,352, 6,204,023, 6,180,370, 5,693,762, 5,530,101, 5,585,089, 5,225,539; and 4,816,567.

Techniques for making partially to fully human antibodies are known in the art and any such techniques can be used. According to one embodiment, fully human antibody sequences are made in a transgenic mouse which has been engineered to express human heavy and light chain antibody genes. Multiple strains of such transgenic mice have been made which can produce different classes of antibodies. B cells from transgenic mice which are producing a desirable antibody can be fused to make hybridoma cell lines for continuous production of the desired antibody. (See for example, Russel, N.D. et al. (2000) Infection and Immunity April 2000:1820-1826; Gallo, M. L. et al. (2000) European J. of Immun. **30**:534-540; Green, L. L. (1999) J. of Immun. Methods **231**:11-23; Yang, X-D et al. (1999A) J. of Leukocyte Biology **66**:401-410; Yang, X-D (1999B) Cancer Research **59**(6):1236-1243; Jakobovits, A. (1998) Advanced Drug Delivery Reviews **31**:33-42; Green, L. and Jakobovits, A. (1998) J. Exp. Med. **188**(3):483-495; Jakobovits, A. (1998) Exp. Opin. Invest. Drugs **7**(4):607-614; Tsuda, H. et al. (1997) Genomics **42**:413-421; Sherman-Gold, R. (1997). Genetic Engineering News **17**(14); Mendez, M. et al. (1997) Nature Genetics **15**:146-156; Jakobovits, A. (1996) WEIR'S HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, THE INTEGRATED IMMUNE SYSTEM VOL. IV, 194.1-194.7; Jakobovits, A. (1995) Current Opinion in Biotechnology **6**:561-566; Mendez, M. et al. (1995) Genomics **26**:294-307; Jakobovits, A. (1994) Current Biology **4**(8):761-763; Arbones, M. et al. (1994) Immunity **1**(4):247-260; Jakobovits, A. (1993) Nature **362**(6417):255-258; Jakobovits, A. et al. (1993) Proc. Natl. Acad. Sci. USA **90**(6):2551-2555; Kucherlapati, et al. U.S. Patent No. 6,075,181.)

Human monoclonal antibodies can also be produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

The antibodies of this invention also can be modified to create chimeric antibodies. Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species. See, *e.g.*, U.S. Patent No.: 4,816,567.

The term "antibody derivative" also includes "diabodies" which are small antibody fragments with two antigen-binding sites, wherein fragments comprise a heavy chain variable domain ($V_H$) connected to a light chain variable domain ($V_L$) in the same polypeptide chain (VH $V_L$). (See for example, EP 404,097; WO 93/11161; and Hollinger et al., (1993) Proc. Natl. Acad. Sci. USA **90**:6444-6448.) By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. (See also, U.S. Patent No. 6,632,926 to Chen et al. which discloses antibody variants that have one or more amino acids inserted into a hypervariable region of the parent antibody and a binding affinity for a target antigen which is at least about two fold stronger than the binding affinity of the parent antibody for the antigen.)

The term "antibody derivative" further includes "linear antibodies". The procedure for making the is known in the art and described in Zapata et al. (1995) Protein Eng. **8**(10):1057-1062. Briefly, these antibodies comprise a pair of tandem Fd segments ($V_H$ -$C_H$ 1-VH -$C_H$1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

The antibodies of this invention can be recovered and purified from recombinant cell cultures by known methods including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography ("HPLC") can also be used for purification.

Antibodies of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a eukaryotic host, including, for example, yeast, higher plant, insect and mammalian cells, or alternatively from a prokaryotic cells as described above.

In some aspects of this invention, it will be useful to detectably or therapeutically label the antibody. Methods for conjugating antibodies to these agents are known in the art. For the purpose of illustration only, antibodies can be labeled with a detectable moiety such as a radioactive atom, a chromophore, a fluorophore, or

the like. Such labeled antibodies can be used for diagnostic techniques, either *in vivo*, or in an isolated test sample. Antibodies can also be conjugated, for example, to a pharmaceutical agent, such as chemotherapeutic drug or a toxin. They can be linked to a cytokine, to a ligand, to another antibody. Suitable agents for coupling to antibodies to achieve an anti-tumor effect include cytokines, such as interleukin 2 (IL-2) and Tumor Necrosis Factor (TNF); photosensitizers, for use in photodynamic therapy, including aluminum (III) phthalocyanine tetrasulfonate, hematoporphyrin, and phthalocyanine; radionuclides, such as iodine-131 ($^{131}$I), yttrium-90 ($^{90}$Y), bismuth-212 ($^{212}$Bi), bismuth-213 ($^{213}$Bi), technetium-99m ($^{99m}$Tc), rhenium-186 ($^{186}$Re), and rhenium-188 ($^{188}$Re); antibiotics, such as doxorubicin, adriamycin, daunorubicin, methotrexate, daunomycin, neocarzinostatin, and carboplatin; bacterial, plant, and other toxins, such as diphtheria toxin, pseudomonas exotoxin A, staphylococcal enterotoxin A, abrin-A toxin, ricin A (deglycosylated ricin A and native ricin A), TGF-alpha toxin, cytotoxin from chinese cobra (naja naja atra), and gelonin (a plant toxin); ribosome inactivating proteins from plants, bacteria and fungi, such as restrictocin (a ribosome inactivating protein produced by *Aspergillus restrictus*), saporin (a ribosome inactivating protein from *Saponaria officinalis*), and RNase; tyrosine kinase inhibitors; ly207702 (a difluorinated purine nucleoside); liposomes containing anti cystic agents (*e.g.*, antisense oligonucleotides, plasmids which encode for toxins, methotrexate, etc.); and other antibodies or antibody fragments, such as F(ab).

With respect to preparations containing antibodies covalently linked to organic molecules, they can be prepared using suitable methods, such as by reaction with one or more modifying agents. Examples of such include modifying and activating groups. A "modifying agent" as the term is used herein, refers to a suitable organic group (*e.g.*, hydrophilic polymer, a fatty acid, a fatty acid ester) that comprises an activating group. Specific examples of these are provided *supra*. An "activating group" is a chemical moiety or functional group that can, under appropriate conditions, react with a second chemical group thereby forming a covalent bond between the modifying agent and the second chemical group. Examples of such are electrophilic groups such as tosylate, mesylate, halo (chloro, bromo, fluoro, iodo), N-hydroxysuccinimidyl esters (NHS), and the like. Activating groups that can react with thiols include, for example, maleimide, iodoacetyl, acrylolyl, pyridyl disulfides, 5-

thiol-2-nitrobenzoic acid thiol (TNB-thiol), and the like. An aldehyde functional group can be coupled to amine- or hydrazide-containing molecules, and an azide group can react with a trivalent phosphorous group to form phosphoramidate or phosphorimide linkages. Suitable methods to introduce activating groups into molecules are known in the art (see for example, Hermanson, G. T., BIOCONJUGATE TECHNIQUES, Academic Press: San Diego, Calif. (1996)). An activating group can be bonded directly to the organic group (*e.g.*, hydrophilic polymer, fatty acid, fatty acid ester), or through a linker moiety, for example a divalent $C_1$-$C_{12}$ group wherein one or more carbon atoms can be replaced by a heteroatom such as oxygen, nitrogen or sulfur. Suitable linker moieties include, for example, tetraethylene glycol. Modifying agents that comprise a linker moiety can be produced, for example, by reacting a mono-Boc-alkyldiamine (*e.g.*, mono-Boc-ethylenediamine, mono-Boc-diaminohexane) with a fatty acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to form an amide bond between the free amine and the fatty acid carboxylate. The Boc protecting group can be removed from the product by treatment with trifluoroacetic acid (TFA) to expose a primary amine that can be coupled to another carboxylate as described, or can be reacted with maleic anhydride and the resulting product cyclized to produce an activated maleimido derivative of the fatty acid.

The modified antibodies of the invention can be produced by reacting a human antibody or antigen-binding fragment with a modifying agent. For example, the organic moieties can be bonded to the antibody in a non-site specific manner by employing an amine-reactive modifying agent, for example, an NHS ester of PEG. Modified human antibodies or antigen-binding fragments can also be prepared by reducing disulfide bonds (*e.g.*, intra-chain disulfide bonds) of an antibody or antigen-binding fragment. The reduced antibody or antigen-binding fragment can then be reacted with a thiol-reactive modifying agent to produce the modified antibody of the invention. Modified human antibodies and antigen-binding fragments comprising an organic moiety that is bonded to specific sites of an antibody of the present invention can be prepared using suitable methods, such as reverse proteolysis. See generally, Hermanson, G. T., BIOCONJUGATE TECHNIQUES, Academic Press: San Diego, Calif. (1996).

## POLYNUCLEOTIDES AND POLYPEPTIDES

Polynucleotides, polypeptides and fragment(s) thereof, can be obtained by using the sequence information provided in Table 1 (and the sequence listing *infra*) and chemical synthesis using a commercially available automated peptide synthesizer such as those manufactured by Perkin Elmer/Applied Biosystems, Inc., Model 430A or 431A, Foster City, CA, USA. The synthesized protein or polypeptide can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Accordingly, this invention also provides a process for chemically synthesizing the proteins of this invention by providing the sequence of the protein and reagents, such as amino acids and enzymes and linking together the amino acids in the proper orientation and linear sequence.

Alternatively, the proteins and polypeptides can be obtained by well-known recombinant methods as described herein using the host cell and vector systems as described herein. The host cell can be prokaryotic or eukaryotic. Host cell systems are described *supra*.

## DIAGNOSTIC METHODS

As noted above, this invention provides various methods for aiding in the diagnosis of the state of a cell that is characterized by abnormal cell growth in the form of, *e.g.*, malignancy, hyperplasia or metaplasia. The methods are particularly useful for aiding in the diagnosis of cancers of epithelial origin, *e.g.*, non-small cell lung cancer (NSCLC), ovarian, breast, prostate and colon cancer. The neoplastic state of a cell can be determined by noting whether the growth of the cell is not governed by the usual limitation of normal growth. For the purposes of this invention, the term also is to include genotypic changes that occur prior to detection of this growth in the form of a tumor and are causative of these phenotypic changes. The phenotypic changes associated with the neoplastic state of a cell (a set of *in vitro* characteristics associated with a tumorigenic ability *in vivo*) include a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, expression of fetal antigens and the like. (*See*, Luria *et al.* (1978) GENERAL VIROLOGY, 3$^{d}$ edition, 436-446 (John Wiley & Sons, New York)).

Accordingly, one embodiment is a method of diagnosing the condition of a cell by screening for the presence of a differentially expressed polynucleotide or polypeptide isolated from a sample containing or suspected of containing having cells that express said gene, in which the differential expression of the gene is indicative of the neoplastic state of the cell. As shown below, the gene is expressed more in a cancer or tumor cell, wherein said cell is one or more of lung, ovarian or prostate, as compared to a counterpart normal or healthy cell or tissue.

Detection can be by any appropriate method, including for example, detecting the quantity of mRNA transcribed from the gene or the quantity of cDNA produced from the reverse transcription of the mRNA transcribed from the gene or the quantity of the polypeptide or protein encoded by the gene. Probes for each of these methods are provided by reverse translating the peptides identified in Table 1 and using the polynucleotides encoding the peptides. These methods can be performed on a sample by sample basis or modified for high throughput analysis. Additionally, databases containing quantitative full or partial transcripts or protein sequences isolated from a cell sample can be searched and analyzed for the presence and amount of transcript or expressed gene product. In one aspect, the database contains at least one of the sequences shown in Table 1 and/or the polynucleotide encoding it.

For the purpose of illustration only, gene expression is determined by noting the amount (if any, *e.g.*, altered) expression of the gene in the test system at the level of an mRNA transcribed from the gene of interest. In a separate embodiment, augmentation of the level of the polypeptide or protein encoded by the gene of interest is indicative of the presence of the neoplastic condition of the cell. The method can be used for aiding in the diagnosis of lung, ovarian or prostate cancer. Thus, by detecting this genotype prior to tumor growth, one can predict a predisposition to cancer and/or provide early diagnosis and treatment.

Cell or tissue samples used for this invention encompass body fluid, solid tissue samples, tissue cultures or cells derived there from and the progeny thereof and sections or smears prepared from any of these sources or any other samples that may contain a cell having a gene described herein. In one embodiment, the sample comprises cells prepared from a subject's tissue, *e.g.*, lung or tissue which may contain a metastatis.

In assaying for an alteration in mRNA level, nucleic acid contained in the aforementioned samples is first extracted according to standard methods in the art. For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook *et al.* (1989) *supra or* extracted by nucleic-acid-binding resins following the accompanying instructions provided by manufactures. The mRNA of a gene contained in the extracted nucleic acid sample is then detected by hybridization (*e.g.*, Northern blot analysis) and/or amplification procedures according to methods widely known in the art or based on the methods exemplified herein.

Nucleic acid molecules having at least 10 nucleotides and exhibiting sequence complementarity or homology to at least one polynucleotide encoding a peptide identified in Table 1 find utility as hybridization probes. It is known in the art that a "perfectly matched" probe is not needed for a specific hybridization. Minor changes in probe sequence achieved by substitution, deletion or insertion of a small number of bases do not affect the hybridization specificity. In general, as much as 20% base-pair mismatch (when optimally aligned) can be tolerated. Preferably, a probe useful for detecting mRNA is at least about 80% identical to the homologous region of comparable size contained in the genes or polynucleotides encoding the peptides identified in Table 1. In one aspect, the probe is 85% identical to the corresponding polynucleotide sequence after alignment of the homologous region or, alternatively, it exhibits 90% identity. These probes can be used in radioassays (*e.g.*, Southern and Northern blot analysis) to detect, prognose, diagnose or monitor various neoplastic states resulting from differential expression of a polynucleotide of interest. The total size of fragment, as well as the size of the complementary stretches, will depend on the intended use or application of the particular nucleic acid segment. Smaller fragments derived from the known sequences will generally find use in hybridization embodiments, wherein the length of the complementary region may be varied, such as between about 10 and about 100 nucleotides or even full-length according to the complementary sequences one wishes to detect.

In one aspect, nucleotide probes having complementary sequences over stretches greater than about 10 nucleotides in length are used, so as to increase stability and selectivity of the hybrid and, thereby, improving the specificity of

particular hybrid molecules obtained. Alternatively, one can design nucleic acid molecules having gene-complementary stretches of more than about 25 or alternatively more than about 50 nucleotides in length or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR™ technology with two priming oligonucleotides as described in U.S. Patent No. 4,603,102 or by introducing selected sequences into recombinant vectors for recombinant production. In one aspect, a probe is about 50 to about 75, nucleotides or, alternatively, about 50 to about 100 nucleotides in length. These probes can be designed from the sequence of full length genes.

In certain embodiments, it will be advantageous to employ nucleic acid sequences as described herein in combination with an appropriate means, such as a label, for detecting hybridization and therefore complementary sequences. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. One can employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

Hybridization reactions can be performed under conditions of different "stringency". Relevant conditions include temperature, ionic strength, time of incubation, the presence of additional solutes in the reaction mixture such as formamide and the washing procedure. Higher stringency conditions are those conditions, such as higher temperature and lower sodium ion concentration, which require higher minimum complementarity between hybridizing elements for a stable hybridization complex to form. Conditions that increase the stringency of a hybridization reaction are widely known and published in the art. *See,* for example, Sambrook *et al.* (1989) *supra.*

The nucleotide probes of the present invention can also be used as primers and detection of genes or gene transcripts that are differentially expressed in certain body

tissues. Additionally, a primer useful for detecting the aforementioned differentially expressed mRNA is at least about 80% identical to the homologous region of comparable size contained in the previously identified sequences encoding the peptides identified in Table 1. For the purpose of this invention, "amplification" means any method employing a primer-dependent polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of *E.coli* DNA polymerase and reverse transcriptase.

A known amplification method is PCR, MacPherson *et al.*, PCR: A PRACTICAL APPROACH, (IRL Press at Oxford University Press (1991)). However, PCR conditions used for each application reaction are empirically determined. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time, $Mg^{2+}$ ATP concentration, pH and the relative concentration of primers, templates and deoxyribonucleotides.

After amplification, the resulting DNA fragments can be detected by agarose gel electrophoresis followed by visualization with ethidium bromide staining and ultraviolet illumination. A specific amplification of differentially expressed genes of interest can be verified by demonstrating that the amplified DNA fragment has the predicted size, exhibits the predicated restriction digestion pattern and/or hybridizes to the correct cloned DNA sequence.

The probes also can be attached to a solid support for use in high throughput screening assays using methods known in the art. PCT WO 97/10365 and U.S. Patent Nos. 5,405,783; 5,412,087 and 5,445,934; for example, disclose the construction of high density oligonucleotide chips which can contain one or more of the sequences disclosed herein. Using the methods disclosed in U.S. Patent Nos. 5,405,783; 5,412,087 and 5,445,934; the probes of this invention are synthesized on a derivatized glass surface. Photoprotected nucleoside phosphoramidites are coupled to the glass surface, selectively deprotected by photolysis through a photolithographic mask and reacted with a second protected nucleoside phosphoramidite. The coupling/deprotection process is repeated until the desired probe is complete.

The expression level of a gene can also be determined through exposure of a nucleic acid sample to a probe-modified chip. Extracted nucleic acid is labeled, for

example, with a fluorescent tag, preferably during an amplification step. Hybridization of the labeled sample is performed at an appropriate stringency level. The degree of probe-nucleic acid hybridization is quantitatively measured using a detection device, such as a confocal microscope. *See,* U.S. Patent Nos. 5,578,832 and 5,631,734. The obtained measurement is directly correlated with gene expression level.

The probes and high density oligonucleotide probe arrays also provide an effective means of monitoring expression of the gene of interest. They are also useful to screen for compositions that upregulate or downregulate the expression of the gene of interest.

In another embodiment, the methods of this invention are used to monitor expression of the gene of interest which specifically hybridize to the probes of this invention in response to defined stimuli, such as an exposure of a cell or subject to a drug.

In one embodiment, the hybridized nucleic acids are detected by detecting one or more labels attached to the sample nucleic acids. The labels may be incorporated by any of a number of means known to those of skill in the art. However, in one aspect, the label is simultaneously incorporated during the amplification step in the preparation of the sample nucleic acid. Thus, for example, polymerase chain reaction (PCR) with labeled primers or labeled nucleotides will provide a labeled amplification product. In a separate embodiment, transcription amplification, as described above, using a labeled nucleotide (*e.g.,* fluorescein-labeled UTP and/or CTP) incorporates a label into the transcribed nucleic acids.

Alternatively, a label may be added directly to the original nucleic acid sample (*e.g.*, mRNA, polyA, mRNA, cDNA, *etc.*) or to the amplification product after the amplification is completed. Means of attaching labels to nucleic acids are known to those of skill in the art and include, for example nick translation or end-labeling (*e.g.*, with a labeled RNA) by kinasing of the nucleic acid and subsequent attachment (ligation) of a nucleic acid linker joining the sample nucleic acid to a label (*e.g.*, a fluorophore).

Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (*e.g.*, Dynabeads™), fluorescent dyes (*e.g.*, fluorescein, Texas red, rhodamine, green fluorescent protein and the like), radiolabels (*e.g.*, $^{3}H$, $^{125}I$, $^{35}S$, $^{14}C$ or $^{32}P$) enzymes (*e.g.*, horseradish peroxidase, alkaline phosphatase and others commonly used in an ELISA) and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex, *etc.*) beads. Patents teaching the use of such labels include U.S. Patents Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Means of detecting such labels are known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate and colorimetric labels are detected by simply visualizing the colored label.

As described in more detail in WO 97/10365, the label may be added to the target (sample) nucleic acid(s) prior to or after the hybridization. These are detectable labels that are directly attached to or incorporated into the target (sample) nucleic acid prior to hybridization. In contrast, "indirect labels" are joined to the hybrid duplex after hybridization. Often, the indirect label is attached to a binding moiety that has been attached to the target nucleic acid prior to the hybridization. Thus, for example, the target nucleic acid may be biotinylated before the hybridization. After hybridization, an avidin-conjugated fluorophore will bind the biotin bearing hybrid duplexes providing a label that is easily detected. For a detailed review of methods of labeling nucleic acids and detecting labeled hybridized nucleic acids; *see,* LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY, Vol. 24: Hybridization with Nucleic Acid Probes, P. Tijssen, ed. Elsevier, N.Y. (1993).

The nucleic acid sample also may be modified prior to hybridization to the high density probe array in order to reduce sample complexity thereby decreasing background signal and improving sensitivity of the measurement using methods known in the art, *e.g.*, the methods disclosed in WO 97/10365.

Results from the chip assay are typically analyzed using a computer software program. *See,* for example, EP 0717 113 A2 and WO 95/20681. The hybridization data is read into the program, which calculates the expression level of the targeted gene(s) *i.e.*, the genes identified in Table 1. This figure is compared against existing data sets of gene expression levels for diseased and healthy individuals. A correlation between the obtained data and that of a set of diseased individuals indicates the onset of a disease in the subject patient.

Also within the scope of this application is a database useful for the detection of neoplastic lung tissue comprising one or more of the sequences, polynucleotides encoding the peptides, or parts thereof, of the peptides listed Table 1.

These polynucleotide sequences are stored in a digital storage medium such that a data processing system for standardized representation of the genes that identify a lung cancer cell is compiled. The data processing system is useful to analyze gene expression between two cells by first selecting a cell suspected of being of a neoplastic phenotype or genotype and then isolating polynucleotides from the cell. The isolated polynucleotides are then sequenced. The sequences from the sample are compared with the sequence(s) present in the database using homology search techniques described above. In one aspect, greater than 90% is selected or, alternatively, greater than 95% is selected or, alternatively, greater than or equal to 97% sequence identity is selected, between the test sequence and at least one sequence, or polynucleotide encoding it, identified in Table 1 or its complement, is a positive indication that the polynucleotide has been isolated from a lung, prostate or ovarian cancer cell as defined above.

Alternatively, one can compare a sample against a database. Briefly, multiple RNAs are isolated from cell or tissue samples using methods known in the art and described for example, in Sambrook *et al.* (1989) *supra.* Optionally, the gene transcripts can be converted to cDNA. A sampling of the gene transcripts are subjected to sequence-specific analysis and quantified. These gene transcript

sequence abundances are compared against reference database sequence abundances including normal data sets for diseased and healthy patients. The patient has the disease(s) with which the patient's data set most closely correlates which includes the overexpression of the transcripts identified herein.

Differential expression of the gene of interest can also be determined by examining the protein product. A variety of techniques are available in the art for protein analysis. They include but are not limited to radioimmunoassays, ELISA (enzyme linked immunoradiometric assays), "sandwich" immunoassays, immunoradiometric assays, *in situ* immunoassays (using *e.g.*, colloidal gold, enzyme or radioisotope labels), western blot analysis, immunoprecipitation assays, immunofluorescent assays and PAGE-SDS. One means to determine protein level involves (a) providing a biological sample containing polypeptides; and (b) measuring the amount of any immunospecific binding that occurs between an antibody reactive to the expression product of a gene of interest and a component in the sample, in which the amount of immunospecific binding indicates the level of the expressed proteins.

Antibodies that specifically recognize and bind to the protein products of these genes are required for these immunoassays. These may be purchased from commercial vendors or generated and screened using methods well known in the art. *See,* Harlow and Lane (1988) *supra* and Sambrook *et al.* (1989) *supra.* Alternatively, polyclonal or monoclonal antibodies that specifically recognize and bind the protein product of a gene of interest can be made and isolated using known methods.

In diagnosing malignancy, hyperplasia or metaplasia characterized by a differential expression of genes, one typically conducts a comparative analysis of the subject and appropriate controls. Preferably, a diagnostic test includes a control sample derived from a subject (hereinafter "positive control"), that exhibits the predicted change in expression of a gene of interest and clinical characteristics of the malignancy or metaplasia of interest. Alternatively, a diagnosis also includes a control sample derived from a subject (hereinafter "negative control"), that lacks the clinical characteristics of the neoplastic state and whose expression level of the gene at question is within a normal range. A positive correlation between the subject and the positive control with respect to the identified alterations indicates the presence of

or a predisposition to said disease. A lack of correlation between the subject and the negative control confirms the diagnosis. In a preferred embodiment, the method is used for diagnosing cancers of epithelial origin, *e.g.*, lung, ovarian or prostate, on the basis of a differential expression of the gene of interest.

## SCREENING ASSAYS

The present invention also provides a screen for identifying leads, drugs, therapeutic biologics and methods for reversing the neoplastic condition of the cells or selectively inhibiting growth or proliferation of the cells described above. In one aspect, the screen identifies lead compounds or biological agents which are useful for the treatment of malignancy, hyperplasia or metaplasia characterized by differential expression of the gene of interest.

Thus, to practice the method *in vitro*, suitable cell cultures or tissue cultures are first provided. The cell can be a cultured cell or a genetically modified cell which differentially expresses the gene of interest associated with a neoplastic cell. Alternatively, the cells can be from a tissue biopsy. The cells are cultured under conditions (temperature, growth or culture medium and gas ($CO_2$)) and for an appropriate amount of time to attain exponential proliferation without density dependent constraints. It also is desirable to maintain an additional separate cell culture; one which does not receive the agent being tested as a control.

As is apparent to one of skill in the art, the method can be modified for high throughput analysis and suitable cells may be cultured in microtiter plates and several agents may be assayed at the same time by noting genotypic changes, phenotypic changes and/or cell death.

When the agent is a composition other than a DNA or RNA nucleic acid molecule, the suitable conditions comprise directly added to the cell culture or added to culture medium for addition. As is apparent to those skilled in the art, an "effective" amount must be added which can be empirically determined.

The screen involves contacting the agent with a test cell characterized by differential expression of the gene of interest and then assaying the cell for the level of the gene of interest expression. In some aspects, it may be necessary to determine the level of the gene of interest expression prior to the assay. This provides a base

line to compare expression after administration of the agent to the cell culture. In another embodiment, the test cell is a cultured cell from an established cell line that differentially expresses a gene of interest. An agent is a possible therapeutic agent if gene expression is returned (reduced or increased) to a level that is present in a cell in a normal or non-neoplastic state, or the cell selectively dies, or exhibits reduced rate of growth.

In yet another aspect, the test cell or tissue sample is isolated from the subject to be treated and one or more potential agents are screened to determine the optimal therapeutic and/or course of treatment for that individual patient.

For the purposes of this invention, an "agent" is intended to include, but not be limited to a biological or chemical compound such as a simple or complex organic or inorganic molecule, a peptide, a protein or an oligonucleotide. A vast array of compounds can be synthesized, for example oligomers, such as oligopeptides and oligonucleotides and synthetic organic compounds based on various core structures; these compounds are also included in the term "agent". In addition, various natural sources can provide compounds for screening, such as plant or animal extracts and the like. It should be understood, although not always explicitly stated that the agent is used alone or in combination with another agent, having the same or different biological activity as the agents identified by the inventive screen. The agents and methods also are intended to be combined with other therapies.

As used herein, the term "reversing the neoplastic state of the cell" is intended to include apoptosis, necrosis or any other means of preventing cell division, reduced tumorigenicity, loss of pharmaceutical resistance, maturation, differentiation or reversion of the neoplastic phenotypes as described herein. As noted above, lung cells having differential expression of a gene of interest that results in the neoplastic state are suitably treated by this method. These cells can be identified by any method known in the art that allows for the identification of differential expression of the gene.

When the agent is a nucleic acid, it can be added to the cell cultures by methods known in the art, which includes, but is not limited to calcium phosphate precipitation, microinjection or electroporation. Alternatively or additionally, the nucleic acid can be incorporated into an expression or insertion vector for

incorporation into the cells. Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art and briefly described *infra*.

Polynucleotides are inserted into vector genomes using methods well known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of restricted polynucleotide. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well-known and available in the art.

One can determine if the object of the method, *i.e.*, reversal of the neoplastic state of the cell, has been achieved by a reduction of cell division, differentiation of the cell or assaying for a reduction in gene overexpression. Cellular differentiation can be monitored by histological methods or by monitoring for the presence or loss of certain cell surface markers, which may be associated with an undifferentiated phenotype, *e.g.*, the expression of the gene of interest.

Kits containing the agents and instructions necessary to perform the screen and *in vitro* method as described herein also are claimed.

When the subject is an animal such as a rat or mouse, the method provides a convenient animal model system which can be used prior to clinical testing of the therapeutic agent or alternatively, for lead optimization. In this system, a candidate agent is a potential drug if gene expression is returned to a normal level or if symptoms associated or correlated to the presence of cells containing differential

expression of a gene of interest are ameliorated, each as compared to untreated, animal having the pathological cells. It also can be useful to have a separate negative control group of cells or animals which are healthy and not treated, which provides a basis for comparison.

## THERAPEUTIC METHODS

Therapeutic agents provided by this invention, include, but are not limited to small molecules, polynucleotides, peptides, antibodies, antigen presenting cells and include immune effector cells that specifically recognize and lyse cells expressing the gene of interest. One can determine if a subject or patient will be beneficially treated by the use of agents by screening one or more of the agents against tumor cells isolated from the subject or patient using methods known in the art. Additional methods are provided *infra*.

In one embodiment, the therapeutic agent is administered in an amount effective to treat cancer of epithelial origin, *e.g.*, lung, ovarian, breast, prostate and colon cancers. Therapeutics of the invention can also be used to prevent progression from a pre-neoplastic or non-malignant state into a neoplastic or a malignant state.

Various delivery systems are known and can be used to administer a therapeutic agent of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis (*See e.g.*, Wu and Wu (1987) J. Biol. Chem. **262**:4429-4432), construction of a therapeutic nucleic acid as part of a retroviral or other vector, *etc.* Methods of delivery include but are not limited to intra-arterial, intra–muscular, intravenous, intranasal and oral routes. In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by injection or by means of a catheter.

The agents identified herein as effective for their intended purpose can be administered to subjects or individuals susceptible to or at risk of developing a disease correlated to the differential expression of the gene of interest. When the agent is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically

administered to the subject. In one aspect, to determine patients that can be beneficially treated, a tumor sample is removed from the patient and the cells are assayed for the differential expression of the gene of interest. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the efficacy and toxicity of the agent. When delivered to an animal, the method is useful to further confirm efficacy of the agent. As an example of an animal model, groups of nude mice (Balb/c NCR nu/nu female, Simonsen, Gilroy, CA) are each subcutaneously inoculated with about $10^5$ to about $10^9$ hyperproliferative, cancer or target cells as defined herein. When the tumor is established, the agent is administered, for example, by subcutaneous injection around the tumor. Tumor measurements to determine reduction of tumor size are made in two dimensions using venier calipers twice a week. Other animal models may also be employed as appropriate.

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

The agents and compositions of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

The pharmaceutical compositions can be administered orally, intranasally, parenterally or by inhalation therapy, and may take the form of tablets, lozenges, granules, capsules, pills, ampoules, suppositories or aerosol form. They may also take the form of suspensions, solutions and emulsions of the active ingredient in aqueous or nonaqueous diluents, syrups, granulates or powders. In addition to an agent of the present invention, the pharmaceutical compositions can also contain other pharmaceutically active compounds or a plurality of compositions of the invention.

More particularly, an agent of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parental (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient and the disease being treated.

Ideally, the agent should be administered to achieve peak concentrations of the active compound at sites of disease. This may be achieved, for example, by the intravenous injection of the agent, optionally in saline or orally administered, for example, as a tablet, capsule or syrup containing the active ingredient. Desirable blood levels of the agent may be maintained by a continuous infusion to provide a therapeutic amount of the active ingredient within disease tissue. The use of operative combinations is contemplated to provide therapeutic combinations requiring a lower total dosage of each component antiviral agent than may be required when each individual therapeutic compound or drug is used alone, thereby reducing adverse effects.

While it is possible for the agent to be administered alone, it is preferable to present it as a pharmaceutical formulation comprising at least one active ingredient, as defined above, together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic agents. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Formulations include those suitable for oral, rectal, nasal, topical (including transdermal, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or suspension in an aqueous or non-aqueous liquid; or as an oil-in–water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented a bolus, electuary or paste.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (*e.g.*, povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (*e.g.*, sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

Pharmaceutical compositions for topical administration according to the present invention may be formulated as an ointment, cream, suspension, lotion, powder, solution, past, gel, spray, aerosol or oil. Alternatively, a formulation may comprise a patch or a dressing such as a bandage or adhesive plaster impregnated with active ingredients and optionally one or more excipients or diluents.

If desired, the aqueous phase of the cream base may include, for example, at least about 30% w/w of a polyhydric alcohol, *i.e.*, an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol,

glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound which enhances absorption or penetration of the agent through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

The oily phase of the emulsions of this invention may be constituted from known ingredients in a known manner. While this phase may comprise merely an emulsifier (otherwise known as an emulgent), it desirably comprises a mixture of at lease one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier which acts as a stabilizer. It is also preferred to include both an oil and a fat. Together, the emulsifier(s) with or without stabilizer(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

Emulgents and emulsion stabilizers suitable for use in the formulation of the present invention include Tween 60, Span 80, cetostearyl alcohol, myristyl alcohol, glyceryl monostearate and sodium lauryl sulphate.

The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties, since the solubility of the active compound in most oils likely to be used in pharmaceutical emulsion formulations is very low. Thus the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters known as Crodamol CAP may be used, the last three being preferred esters. These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Formulations suitable for topical administration to the eye also include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent for the agent.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the agent, such carriers as are known in the art to be appropriate.

Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 20 to about 500 microns which is administered in the manner in which snuff is taken, *i.e.*, by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops or by aerosol administration by nebulizer, include aqueous or oily solutions of the agent.

Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents, thickening agents and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily subdose, as herein above-recited, or an appropriate fraction thereof, of an agent.

**TRANSGENIC ANIMALS**

In another aspect, the gene of interest can be used to generate transgenic animal models. In recent years, geneticists have succeeded in creating transgenic animals, for example mice, by manipulating the genes of developing embryos and introducing foreign genes into these embryos. Once these genes have integrated into

the genome of the recipient embryo, the resulting embryos or adult animals can be analyzed to determine the function of the gene. The mutant animals are produced to understand the function of known genes *in vivo* and to create animal models of human diseases. (*See e.g.*, Chisaka *et al.* (1992) **355**:516-520; Joyner *et al.* (1992) in POSTIMPLANTATION DEVELOPMENT IN THE MOUSE (Chadwick and Marsh, eds., John Wiley & Sons, United Kingdom) pp:277-297; Dorin *et al.* (1992) Nature **359**:211-215).

U.S. Patent Nos. 5,464,764 and 5,487,992 describe one type of transgenic animal in which the gene of interest is deleted or mutated sufficiently to disrupt its function. (*See also,* U.S. Patent Nos. 5,631,153 and 5,627,059). These "knock-out" animals, made by taking advantage of the phenomena of homologous recombination, can be used to study the function of a particular gene sequence *in vivo*. The polynucleotide sequences described herein are useful in preparing animal models of lung cancer.

## EXPERIMENTAL METHODS

### Experiment No. 1: Expression Analysis

Non-small cell lung cancer globally represents a huge unmet medical need. Fresh non-necrotic NSCLC tumor tissue and corresponding normal tissue from individual patients free of infectious disease were obtained. Fresh tissue samples were at least 1.5 grams of tissue less than 24 hrs from surgery shipped on wet ice. Prior to analysis, the tissue was assessed by a pathologist for percent tumor content, tumor stage and histological type. All of the tissues received were primary lung cancer of stage I, II or IIIa where surgery is the first or primary treatment.

Upon receipt of the tissues, the samples the tissues were minced with crossed scalpels. The minced tissues with treated with collagenase and elastase until single cell suspensions were obtained. The single cell suspensions were washed several times and the red blood cells were lysed. The white blood cells were removed by exposure of the cell suspension to antibodies to CD64, CD45 and CD14 linked to magnetic beads. The epithelial cells were isolated using an antibody to BerEP4 linked to a magnetic bead. The endothelial cells were isolated using an antibody to CD31 linked to a magnetic bead. RNA was prepared from the epithelial cell and endothelial cell samples immediately.

The quality of the RNA from the epithelial and endothelial cells was assessed overall and by expression of specific markers for the cell types. Cytokeratin 18, von Willebrand factor, EF1, P1H12, hevin and cytokeratin 8 were used as markers to determine whether the RNA collected represented a relatively pure cell population of epithelial cells or endothelial cells. After the quality assessment of the RNAs, 4 squamous carcinoma samples, 2 adenocarcinoma samples and 3 normal lung tissue samples were of sufficient quantity and quality for SAGE analysis.

LongSAGE™ was performed on the 9 RNA samples to a depth of approximately 50,000 tags for each library using the methods disclosed in Nature Biotechnology (2002) **20**:508-512. An in-depth bioinformatics analysis was undertaken to characterize the SAGE data based upon increased expression of mRNAs across tumor types and in the squamous and adenocarcinoma compared with the normal lung cells. The focus for potential antibody therapeutic targets was on proteins expressed in the plasma membrane.

GITR, glucocorticoid-induced TNFR-related protein, is known to be expressed by activated T cells and Treg cells. GITR is also known as activation-inducible TNFR family receptor (AITR) and tumor necrosis factor receptor superfamily, member 18 (TNFRSF-18) (Stephens, G.L. *et al.* (2004) J. Immunol. **173**:5008-5020 and Nocentini, G. *et al.* (1997) P.N.A.S. **94**:6216-6221). GITR ligand binds to GITR and triggers NF-kappaB activation through TRAF2. The GITR –GITR ligand interaction interrupts TCR-CD3 activation – induced apoptosis in T cells and may be involved in cell survival. The GITR ligand is also known as AITRL, GITRL, TL6 and hGIRTL. GITR is a 228 amino acid transmembrane protein that is suggested to be similar to 4-1BB and CD27. GITR protein has a 19 amino acid signal sequence, 134 amino acid extracellular region with three cysteine-rich motifs, a 23 amino acid transmembrane segment and a 52 amino acid cytoplasmic domain. The GITR ligand is expressed by endothelial cells (including HUVEC), B1 lymphocytes, mature and immature dendritic cells, and macrophages (Stephens, G. *et al.* (2004) *supra.*). GITR is involved in the interactions between T-lymphocytes and endothelial cells and in the regulation of T-cell receptor-mediated cell death. GITR mediates NF-kappaB activation via the TRAF2/NIK pathway. GITR binds to TNF receptor-associated

factor-1 (TRAF1), TRAF2 and TRAF3 but not to TRAF5 and TRAF6 (Nocentini, G. *et al.* (1997) *supra.*).

GITR is expressed on CD4+CD25+ T cells and after interaction with GITRL down-regulates T regulatory suppressor activity. Targeting GITR on tumor cells and depletion of CD4+CD25+ T cells could potentiate the efficacy of active tumor specific therapy (Kohm, A.P. *et al.* (2004) J. Immunol. **172**:4686-4690 and Shimizu, J. *et al.* (2002) Nature Immunol. **3**:135-142).

RT-PCR of bulk tissue RNA from 55 lung tumors and 18 normal lung tissues indicated ≥2-fold increased levels of GITR RNA in 76% of tumors compared with normal tissues. By RT-PCR GITR has very minimal expression in a variety of normal tissues including breast, prostate, brain, heart, kidney, liver, salivary gland, spleen stomach, thymus and uterus. RT-PCR of bulk tissues RNA from a variety of tumors and corresponding normal tissues indicated ≥2-fold increased levels of GITR RNA in 50% of ovarian cancers (n=40), 25% of melanomas (n=22), 50% of prostate cancers (n=24), 20% of colon cancers (n=26), and 66% of breast cancers (n=23).

Immunohisochemistry was performed on formalin-fixed paraffin-embedded human non-small cell lung cancer specimens. The antibodies used were anti-GITR (Research Systems, BA689), anti-RDC1 (Lifescience, RDC1-LP1439). CD31 (DAKO, M0823), anti-alpha-smooth muscle actin (DAKO, M0851), anti-epthelial membrane antigen (DAKO, M0804) and wide spectrum cytokeratin (DAKO, Z0622).

Overall, there was strong GITR reactivity on tumor cells of both adenocarcinoma and squamous carcinoma of the lung. There was also strong reactivity on infiltrating cells within the tumor stroma of both adenocarcinoma and squamous carcinoma of the lung. There was strong RDC1 reactivity on tumor cells of both adenocarcinoma and squamous carcinoma of the lung. There was moderate RDC1 reactivity on endothelial cells and pericytes/smooth muscle cells associated with the tumor vasculature.

By fluorescence activated cytometry (FACS), the NCI-H358 and NCI-H1436 cell lines have very good expression of GITR, and NCI-H1299, NCI-H522, NCI-H23 and NCI-H647 have good expression of GITR. By FACS the expression of GITR on

activated PBMC and activated CD3+ T cells is much lower than is the expression on non-small cell lung cancer cell lines.

**Experiment No. 2: Functional BioAssays**

After generation the panel of antibodies are screened using cell based assays to identify those which neutralize the function of the target protein and reverse the malignant phenotype using methods known in the art. For the purpose of illustration, such methods are described in: Stanton, C.A. *et al.* (2004) Blood **103(2)**:601-606 and Malinda, K.M. *et al.* (1999) Exp. Cell Res. **250**:168-173 (migration assay); paragraphs [0210] through [0226] of U.S. Patent Publication No. 2004/0253708A1 (apoptosis); paragraphs [0183] through [0194] of U.S. Patent Publication No. 2004/0258685A1 (inhibition, anti-proliferation, blocking and epitope binding assays); Stanton, C.A. *et al.* (2004) *supra* (proliferation and cytotoxicity assays); Manches, O. *et al.* (2003) Blood **101(3)**:949-954 (apoptosis, phagocytosis and ADCC assays); and paragraph [0066] of U.S. Patent Publication 2004/0228859A1 (CDC assay).

**Experiment No. 3: *In vivo* Efficacy**

Antibodies that possess the requisite bio-activity as described for example, in Experiment No. 2, are then further screened for *in vivo* efficacy using a syngeneic tumor model or human tumor xenographt model. Such assays and models are known to those of skill in the art. For the purpose of illustration, such methods and described in Tumor Models in Cancer Research, Teicher, B.A. ed. in the series Cancer Drug Discovery and Development, Humana Press, 2004 and Lev. A. *et al.* (2004) PNAS **101(24)**:9051-9056.

Although the above experiments and detailed description are described in reference to use against NSCLC, it should be apparent to those of skill in the art that the methods and compositions of this invention are relevant to the other cancers identified in Table 1. Thus, this invention provides methods and compositions to diagnose and prognose these malignancies as well.

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the following examples are intended to illustrate and not limit the scope of the invention.

Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

# CLAIMS

What is claimed is:

1. A method for diagnosing or prognosing a cancer selected from the group consisting of non-small cell lung cancer (NSCLC), ovarian, breast, prostate and colon cancer, comprising detecting the level of expression of a gene or gene product identified in Table 1 or fragment thereof in a subject, wherein an overexpression of the gene or gene product is a predictive positive diagnosis or prognosis of said cancer in the subject.

2. The method of claim 1, wherein the level of expression of the gene or gene product or fragment thereof is determined immunochemically.

3. The method of claim 2, wherein the level of expression of the gene or gene product or fragment thereof is determined immunochemically by an antibody.

4. The method of claim 1, wherein the level of expression of the gene or gene product or fragment thereof is determined immunochemically by a monoclonal antibody, variant or derivative thereof.

5. The method of claim 1, wherein the level of expression of the gene or gene product or fragment thereof is determined by an antibody selected from the group consisting of a monoclonal antibody, a polyclonal antibody, an antibody variant, an antibody derivative, a humanized antibody and an antibody fragment.

6. The method of claim 1, wherein the level of expression of the gene or gene product is determined by detecting the amount of polynucleotide.

7. The method of claim 1, wherein the detecting is performed on a suitable sample isolated from the subject.

8. The method of claim 7, wherein the suitable sample is preserved tissue sample, a tissue biopsy sample or a sample of body fluid.

9. The method of claim 7, wherein the suitable sample is a sample of body fluid selected from the group consisting of urine, blood and serum.

10. The method of claim 6, wherein the polynucleotide is selected from the group consisting of mRNA and cDNA.

SEQUENCE LISTING

SEQ. ID NO.: 1

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1   atggcacagc acggggcgat gggcgcgttt cgggccctgt gcggcctggc gctgctgtgc
61  gcgctcagcc tgggtcagcg ccccaccggg ggtcccgggt gcggccctgg gcgcctcctg
121 cttgggacgg gaacggacgc gcgctgctgc cgggttcaca cgacgcgctg ctgccgcgat
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SEQUENCE LISTING

<110> Genzyme Corporation
Teicher, Beverly A.
Roberts, Bruce L.
Shankara, Srinivas

<120> GITR Antibodies for the diagnosis of NSCLC

<130> 061698-2010

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<141> 2006-01-19

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| | | | |
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| 专利名称(译) | 用于诊断nsclc的GITR抗体 | | |
| 公开(公告)号 | EP1856281A2 | 公开(公告)日 | 2007-11-21 |
| 申请号 | EP2006719028 | 申请日 | 2006-01-19 |
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| IPC分类号 | C12Q1/68 G01N33/53 | | |
| CPC分类号 | C12Q1/6886 C12Q2600/118 C12Q2600/136 C12Q2600/158 | | |
| 代理机构(译) | LUCEY，MICHAEL | | |
| 优先权 | 60/645349 2005-01-19 US | | |
| 外部链接 | Espacenet | | |


摘要(译)

本发明提供了用于检测，诊断，预测和监测上皮来源的癌症（例如NSCLC）的进展的方法和组合物，所述癌症例如肺癌，卵巢癌，乳腺癌，前列腺癌和结肠癌以及用于所述方法的恶性肿瘤和试剂盒。还提供了用于筛选以鉴定与这些癌症和恶性肿瘤相关的抗原的激动剂和拮抗剂的方法。