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(54) **Title**: COMPOSITIONS AND METHODS FOR THE THERAPY AND DIAGNOSIS OF CANCER

(57) **Abstract**: Compositions and methods for the therapy and diagnosis of cancer are disclosed. For example, illustrative compositions comprise one or more cancer- associated antibodies, polypeptides, polynucleotides, antigen presenting cells, and the like. The disclosed compositions are useful, for example, in the diagnosis, prevention and/or treatment of diseases, particularly cancer.

COMPOSITIONS AND METHODS FOR THE THERAPY AND DIAGNOSIS OF CANCER

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/583,033, filed January 4, 2012; U.S. Provisional Application No. 61/547,342, filed October 14, 2011; and U.S. Provisional Application No. 61/499,534, filed June 21, 2011, each of which is incorporated by reference herein in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is ONCF_001_03WO_ST25.txt. The text file is 88 KB, was created on June 19, 2012, and is being submitted electronically via EFS-Web.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to therapy and diagnosis of cancer. The invention is more specifically related to pharmaceutical and diagnostic compositions comprising antibodies and antigen-binding fragments that specifically bind to cancer-associated proteins (*e.g.,* oncofactors). The invention further relates to pharmaceutical and diagnostic compositions comprising cancer-associated polynucleotides, polypeptides, expression vectors, host cells and the like.

Description of the Related Art

Cancer is a significant health problem throughout the world. Although advances have been made in detection and therapy of cancer, no universally successful method for prevention and/or treatment is currently available. Current therapies, which are generally based on a combination of chemotherapy, surgery and/or radiation, are relatively non-selective and continue to prove inadequate in many patients.

Chemotherapy, in particular, results in numerous side effects, in some cases so severe as to limit the dosage that can be given and thus preclude the use of potentially effective drugs. Moreover, cancers often develop resistance to chemotherapeutic drugs.

In spite of considerable research, there remain great obstacles in effective diagnosis and treatment of many human cancer types. Accordingly, there remains a need in the art for alternative and improved methods for detecting and treating such cancers. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

According to one aspect of the present invention, there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an isolated antibody or antigen-binding fragment thereof that specifically binds a sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2), IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO: 19), SEMA4B (SEQ ID NO: 20), SEMA4D (SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24).In another aspect, an endotoxin-free pharmaceutical composition comprising a pharmaceutically acceptable carrier and an isolated antibody or antigen-binding fragment thereof that specifically binds a sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2), IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO: 19), SEMA4B (SEQ ID NO: 20), SEMA4D

(SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24) is provided.

In a further aspect, a pharmaceutical composition formulated for intravenous injection for use in a patient having or at risk for having cancer, said composition comprising a pharmaceutically acceptable carrier and an isolated antibody or antigen-binding fragment thereof that specifically binds a sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2), IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO: 19), SEMA4B (SEQ ID NO: 20), SEMA4D (SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24).

In a particular aspect, a composition comprises one or more antibodies or antigen binding fragments thereof, wherein each of the one or more antibodies or antigen binding fragments thereof specifically bind a sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2), IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO: 19), SEMA4B (SEQ ID NO: 20), SEMA4D (SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24).

In a certain aspect, a composition is 95%, 96%, 97%, 98%, or 99% endotoxin free.

In a more specific embodiment of the invention, an isolated antibody or antigen-binding fragment of the invention is a monoclonal antibody or antigen-binding

fragment.

In another specific embodiment of the invention, an isolated antibody or antigen-binding fragment of the invention is a humanized antibody or antigen-binding fragment.

In still another specific embodiment, an antibody or antigen binding fragment of the invention is conjugated to a toxin, including, without limitation, a ricin toxin, abrin toxin, diptheria toxin, cholera toxin, gelonin toxin, Pseudomonas exotoxin, Shigella toxin, and pokeweed antiviral protein.

In another specific embodiment, an antibody or antigen-binding fragment of the invention is conjugated to a radionuclide, including, without limitation, ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{211}At and ^{212}Bi.

According to another aspect of the invention, there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an isolated polypeptide of any one of SEQ ID NOs: 1-24, or a fragment or variant thereof having at least 70%, 80%, 90% or 95% identity thereto, or an isolated polynucleotide encoding any one of the foregoing polypeptides. Of course, it will be recognized that additional components may be present in the pharmaceutical compositions of the invention, such as immunostimulants and the like. It will also be recognized, in the context of embodiments employing polynucleotides of the invention that the polynucleotides may be present, for example, in expression vectors, host cells and the like.

According to another aspect of the invention, there is provided a method for the treatment of cancer in a subject in need thereof comprising administering to the subject a pharmaceutical composition as described according to the present invention. The cancer to be treated can be essentially any cancer type with which a sequence of the invention is associated, including, without limitation, cancers of the liver, pancreas, lung, breast, bladder, kidney, and skin (*e.g.*, melanoma), as well as hematological cancers (*e.g.*, leukemia, lymphoma, *etc.*).

The present invention, in another aspect, provides methods relating to the use of an isolated antibody or antigen-binding fragment that specifically binds to a sequence set forth in any one of SEQ ID NOs: 1-24, in the manufacture of a

medicament for the treatment of cancer.

The present invention, in another aspect, provides methods relating to the use of an isolated polypeptide comprising a sequence set forth in any one of SEQ ID NOs: 1-24, or a fragment or variant thereof having at least 90% identity thereto, in the manufacture of a medicament for the treatment of cancer.

The present invention, in another aspect, provides methods relating to the use of an isolated polynucleotide encoding an amino acid sequence set forth in any one of SEQ ID NOs: 1-24, or encoding a fragment or variant of a sequence set forth in SEQ ID NOs: 1-24 having at least 90% identity thereto, in the manufacture of a medicament for the treatment of cancer.

The present invention, in another aspect, provides methods relating to the use of an oligonucleotide that is complementary to a polynucleotide encoding an amino acid sequence set forth in any one of SEQ ID NOs: 1-24, or encoding a fragment or variant of a sequence set forth in SEQ ID NOs: 1-24 having at least 90% identity thereto, in the manufacture of a medicament for the treatment of cancer. In certain embodiments, the oligonucleotide is an antisense oligonucleotide, an RNAi molecule, a ribozyme, or another inhibitory nucleic acid molecule.

According to yet another aspect of the present invention, there is provided a method for detecting the presence of a cancer in a patient, comprising the steps of: (a) obtaining a biological sample from the patient; (b) contacting the biological sample with an antibody or antigen-binding fragment that specifically binds to a polypeptide of any one of SEQ ID NOs: 1-24; (c) detecting in the sample an amount of polypeptide that binds to the antibody or antigen-binding fragment; and (d) comparing the amount of polypeptide to a predetermined cut-off value and therefrom determining the presence of a cancer in the patient.

According to still another aspect of the invention, there is provided a method for detecting the presence of a cancer in a patient, comprising the steps of: (a) obtaining a biological sample from the patient; (b) contacting the biological sample with an oligonucleotide that hybridizes to a polynucleotide, wherein the polynucleotide encodes a polypeptide of any one of SEQ ID NOs: 1-24, or encodes a fragment or variant thereof having at least 90% identity thereto; (c) detecting in the sample an

amount of a polynucleotide that hybridizes to the oligonucleotide; and (d) comparing the amount of polynucleotide that hybridizes to the oligonucleotide to a predetermined cut-off value, and therefrom determining the presence of the cancer in the patient.

In still another aspect, the present invention provides a diagnostic kit comprising at least one isolated antibody or antigen-binding fragment thereof that specifically binds to a sequence of any one of SEQ ID NOs: 1-24 and a detection reagent, wherein the detection reagent comprises a reporter group.

In still another aspect, the present invention provides a diagnostic kit comprising at least one oligonucleotide that hybridizes to a polynucleotide, wherein the polynucleotide encodes a polypeptide of any one of SEQ ID NOs: 1-24, or encodes a fragment or variant thereof having at least 90% identity thereto.In one aspect, a method is provided for treating a cancer in a patient, comprising the steps of: (a) detecting an amount of polypeptide of any one of SEQ ID NOs: 1-24 in a biological sample of a patient; (b) comparing the amount of the polypeptide to a predetermined cut-off value and therefrom determining the presence of a cancer in the patient; and (c) administering a composition according to any one of claims 1 to 14 to a subject determined to have cancer in step (b).

In a certain aspect, a method is provided for treating a cancer in a patient, comprising the steps of: (a) obtaining a biological sample from the patient; (b) contacting the biological sample with an oligonucleotide that hybridizes to a polynucleotide, wherein the polynucleotide encodes a polypeptide of any one of SEQ ID NOs: 1-24, or encodes a fragment or variant thereof having at least 90% identity thereto; (c) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide; (d) comparing the amount of polynucleotide that hybridizes to the oligonucleotide to a predetermined cut-off value, and therefrom determining the presence of the cancer in the patient; and (e) administering a composition according to any one of claims 1 to 14 to a subject determined to have cancer in step (d).

In another aspect, the cancer is selected from liver cancer, pancreatic cancer, lung cancer, breast cancer, bladder cancer, kidney cancer, skin cancer and hematological cancer.These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings.

All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the results from a representative mixed tumor lymphocyte culture (MTLC) assay. Raji B lymphoma cells were cocultured with PBMCs and treated with various concentrations of recombinant IL1f5 polypeptide.

Figure 2 shows the results from a representative MTLC assay. Raji B lymphoma cells were cocultured with PBMCs and treated with various concentrations of recombinant IL1RAP2 polypeptide.

Figure 3 shows the results from a representative MTLC assay. Raji B lymphoma cells were cocultured with PBMCs and treated with various concentrations of recombinant CCL14 polypeptide.

Figure 4 shows the results from a representative immunohistochemistry (IHC) assay. IL1f5 expression in invasive ductal carcinoma breast cancer cells is increased compared to IL1f5 expression in normal ductal epithelium, vessels, and stroma.

Figure 5 shows the results from a representative IHC assay. IL1f5 expression in adenocarcinoma colon cancer cells is increased compared to IL1f5 expression in normal colonic tissue.

Figure 6 shows the results from a representative IHC assay. IL1f5 expression in adenocarcinoma prostate cancer cells is increased compared to IL1f5 expression in normal prostate gland and stroma.

Figure 7 shows the results from a representative IHC assay. IL1f5 expression in squamous cell carcinoma, adenocarcinoma, and papillary adenocarcinoma lung cancer cells is increased compared to IL1f5 expression in normal alveolar tissue.

Figure 8 shows the results from a representative IHC assay. GPR183 expression in invasive ductal carcinoma breast cancer cells is increased compared to GPR183 expression in normal ductal epithelium, vessels, and stroma.

Figure 9 shows the results from a representative IHC assay. GPR183 expression in adenocarcinoma colon cancer cells is increased compared to GPR183 expression in normal colonic tissue.

Figure 10 shows the results from a representative IHC assay. GPR183 expression in squamous cell carcinoma and adenocarcinoma lung cancer cells is increased compared to GPR183 expression in normal alveolar tissue.

Figure 11 shows the results from a representative IHC assay. IL1RAP expression in invasive ductal carcinoma breast cancer cells is increased compared to IL1RAP expression in normal breast ductal epithelium, vessels, and stroma.

Figure 12 shows the results from a representative IHC assay. IL1RAP expression in lung cancer cells is increased compared to IL1RAP expression in normal lung alveoli.

Figure 13 shows the results from a representative IHC assay. CCL14 expression in invasive ductal carcinoma breast cancer cells is increased compared to CCL14 expression in normal breast ductal epithelium, vessels, and stroma.

Figure 14 shows the results from a representative IHC assay. CCL14 expression in prostate adenocarcinoma is increased compared to CCL14 expression in normal prostate glands and stroma.

Figure 15 shows the results from a representative IHC assay. CCL14 expression in lung cancer cells is increased compared to CCL14 expression in normal lung alveoli.

Figure 16 shows the results from a representative IHC assay. SEMA4D expression in invasive ductal carcinoma breast cancer cells is increased compared to SEMA4D expression in normal breast ductal epithelium, vessels, and stroma.

Figure 17 shows the results from a representative IHC assay. IL1R2 expression in invasive ductal carcinoma breast cancer cells is increased compared to IL1R2 expression in normal breast ductal epithelium, vessels, and stroma.

Figure 18 shows the results from a representative MTLC assay. Raji B lymphoma cells were cocultured with PBMCs and treated with various concentrations of recombinant IL1R2 polypeptide.

Figure 19 shows the results from a representative T cell proliferation assay. Raji B lymphoma cells were cocultured with PBMCs and treated with various concentrations of recombinant IL1f5 polypeptide. The effect of IL1f5 on T cell proliferation was measured.

BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFIERS

SEQ ID NO:1 is an amino acid sequence of the protein IL1f5 (NP_036407.1).

SEQ ID NO:2 is an amino acid sequence of the protein CCBP2 (NP_001287.2).

SEQ ID NO:3 is an amino acid sequence of the protein IL1R2 (NP_004624.1).

SEQ ID NO:4 is an amino acid sequence of the protein IL1RAPL1 (NP_055086.1).

SEQ ID NO:5 is an amino acid sequence of the protein IL18BP (NP_766630.2).

SEQ ID NO:6 is an amino acid sequence of the protein CLEC2B (NP_005118.2).

SEQ ID NO:7 is an amino acid sequence of the protein C4BPA (NP_000706.1).

SEQ ID NO:8 is an amino acid sequence of the protein C4BPB (NP_000707.1).

SEQ ID NO:9 is an amino acid sequence of the protein SERPINI1 (NP_005016.1).

SEQ ID NO:10 is an amino acid sequence of the protein IL1RAP isoform 1 (NP_002173.1).

SEQ ID NO:11 is an amino acid sequence of the protein IL1RAP isoform 2 (NP_608273.1).

SEQ ID NO:12 is an amino acid sequence of the protein GPR1 (NP_005270.2).

SEQ ID NO:13 is an amino acid sequence of the protein GPR4 (NP_005273.1).

SEQ ID NO:14 is an amino acid sequence of the protein GPR15 (NP_005281.1).

SEQ ID NO:15 is an amino acid sequence of the protein GPR32 (NP_001497.1).

SEQ ID NO:16 is an amino acid sequence of the protein GPR34 (NP_005291.1).

SEQ ID NO:17 is an amino acid sequence of the protein GPR183 (NP_004942.1).

SEQ ID NO:18 is an amino acid sequence of the protein SERPINA4 (NP_006206.2).

SEQ ID NO:19 is an amino acid sequence of the protein SERPINB5 (NP_002630.2).

SEQ ID NO:20 is an amino acid sequence of the protein SEMA4B (NP_064595.2).

SEQ ID NO:21 is an amino acid sequence of the protein SEMA4D (NP_006369.3).

SEQ ID NO:22 is an amino acid sequence of the protein CCL14 (NP_116739.1).

SEQ ID NO:23 is an amino acid sequence of the protein NKTR (NP_005376.2).

SEQ ID NO:24 is an amino acid sequence of the protein SFTPD (NP_003010.4).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates generally to pharmaceutical compositions comprising one or more of the oncofactor antibodies, polynucleotides, polypeptides, T-cells and/or other compositions disclosed herein in pharmaceutically-acceptable carriers for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. More specifically, as described herein, the

polynucleotide and polypeptide sequences of the present invention represent oncofactor sequences and are important targets useful in the detection and treatment of cancer. Accordingly, illustrative aspects of the present invention include, but are not restricted to, various uses of the described oncofactor sequences and related binding agents (*e.g.*, antibodies) in the detection and/or treatment of cancer.

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of virology, immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., 1984); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., 1985); *Transcription and Translation* (B. Hames & S. Higgins, eds., 1984); *Animal Cell Culture* (R. Freshney, ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984).

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

Antibodies, Fragments Thereof and Other Binding Agents

As noted, according to one aspect, the present invention provides pharmaceutical compositions comprising binding agents, such as antibodies and antigen-binding fragments thereof, that exhibit immunological binding to a cancer-associated sequence disclosed herein (*e.g.,* oncofactors), or to a portion, variant or derivative thereof.

More specifically, in certain preferred embodiments, the pharmaceutical compositions of the invention comprise antibodies and/or antigen-binding fragments that are capable of specifically binding to a cancer-associated polypeptide sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2),

IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO: 19), SEMA4B (SEQ ID NO: 20), SEMA4D (SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24).

An antibody, or antigen-binding fragment thereof, is said to "specifically bind," "immunogically bind," and/or is "immunologically reactive" to a polypeptide of the invention if it reacts at a detectable level (within, for example, an ELISA assay) with the polypeptide, and does not react detectably with unrelated polypeptides under similar conditions. Immunological binding, as used in this context, generally refers to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (K_{on}) and the "off rate constant" (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of K_{off} /K_{on} enables cancellation of all parameters not related to affinity, and is thus equal to the dissociation constant K_d. See, generally, Davies *et al.* (1990) *Annual Rev. Biochem. 59*:439-473.

An "antigen-binding site," or "binding portion" of an antibody refers to the part of the immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches

within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions," or "FRs". Thus the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs."

In a preferred embodiment, a binding agent is an antibody or an antigen-binding fragment thereof. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.,* Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of recombinant antibodies. In one technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (*e.g.,* mice, rats, rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for an antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol. 6*:511-519, 1976, and improvements thereto. Briefly, these methods involve

the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

A number of therapeutically useful molecules are known in the art which comprise antigen-binding sites that are capable of exhibiting immunological binding properties of an antibody molecule. The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the "F(ab)" fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the "$F(ab')_2$ " fragment which comprises both antigen-binding sites. An "Fv" fragment can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes a

non-covalent V_H::V_L heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. Inbar *et al.* (1972) *Proc. Nat. Acad. Sci. USA 69*:2659-2662; Hochman *et al.* (1976) *Biochem 15*:2706-2710; and Ehrlich *et al.* (1980) *Biochem 19*:4091-4096.

A single chain Fv ("sFv") polypeptide is a covalently linked V_H::V_L heterodimer which is expressed from a gene fusion including V_H- and V_L-encoding genes linked by a peptide-encoding linker. Huston *et al.* (1988) *Proc. Nat. Acad. Sci. USA 85*(16):5879-5883. A number of methods have been described to discern chemical structures for converting the naturally aggregated—but chemically separated—light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, *e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston *et al.*; and U.S. Pat. No. 4,946,778, to Ladner *et al.*

Each of the above-described molecules includes a heavy chain and a light chain CDR set, respectively interposed between a heavy chain and a light chain FR set which provide support to the CDRS and define the spatial relationship of the CDRs relative to each other. As used herein, the term "CDR set" refers to the three hypervariable regions of a heavy or light chain V region. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (*e.g.*, a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site.

As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues

directly adjacent to the CDRS. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures—regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

A number of "humanized" antibody molecules comprising an antigen-binding site derived from a non-human immunoglobulin have been described, including chimeric antibodies having rodent V regions and their associated CDRs fused to human constant domains (Winter *et al.* (1991) *Nature 349*:293-299; Lobuglio *et al.* (1989) *Proc. Nat. Acad. Sci. USA 86*:4220-4224; Shaw *et al.* (1987) *J Immunol. 138*:4534-4538; and Brown *et al.* (1987) *Cancer Res. 47*:3577-3583), rodent CDRs grafted into a human supporting FR prior to fusion with an appropriate human antibody constant domain (Riechmann *et al.* (1988) *Nature 332*:323-327; Verhoeyen *et al.* (1988) *Science 239*:1534-1536; and Jones *et al.* (1986) *Nature 321*:522-525), and rodent CDRs supported by recombinantly veneered rodent FRs (European Patent Publication No. 519,596, published Dec. 23, 1992). These "humanized" molecules are designed to minimize unwanted immunological response toward rodent antihuman antibody molecules which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients.

As used herein, the terms "veneered FRs" and "recombinantly veneered FRs" refer to the selective replacement of FR residues from, *e.g.*, a rodent heavy or light chain V region, with human FR residues in order to provide a xenogeneic molecule comprising an antigen-binding site which retains substantially all of the native FR polypeptide folding structure. Veneering techniques are based on the understanding that the ligand binding characteristics of an antigen-binding site are determined primarily by the structure and relative disposition of the heavy and light chain CDR sets within the antigen-binding surface. Davies *et al.* (1990) *Ann. Rev. Biochem. 59*:439-

473. Thus, antigen binding specificity can be preserved in a humanized antibody only wherein the CDR structures, their interaction with each other, and their interaction with the rest of the V region domains are carefully maintained. By using veneering techniques, exterior (*e.g.*, solvent-accessible) FR residues which are readily encountered by the immune system are selectively replaced with human residues to provide a hybrid molecule that comprises either a weakly immunogenic, or substantially non-immunogenic veneered surface.

The process of veneering makes use of the available sequence data for human antibody variable domains compiled by Kabat *et al.*, in Sequences of Proteins of Immunological Interest, 4th ed., (U.S. Dept. of Health and Human Services, U.S. Government Printing Office, 1987), updates to the Kabat database, and other accessible U.S. and foreign databases (both nucleic acid and protein). Solvent accessibilities of V region amino acids can be deduced from the known three-dimensional structure for human and murine antibody fragments. There are two general steps in veneering a murine antigen-binding site. Initially, the FRs of the variable domains of an antibody molecule of interest are compared with corresponding FR sequences of human variable domains obtained from the above-identified sources. The most homologous human V regions are then compared residue by residue to corresponding murine amino acids. The residues in the murine FR which differ from the human counterpart are replaced by the residues present in the human moiety using recombinant techniques well known in the art. Residue switching is only carried out with moieties which are at least partially exposed (solvent accessible), and care is exercised in the replacement of amino acid residues which may have a significant effect on the tertiary structure of V region domains, such as proline, glycine and charged amino acids.

In this manner, the resultant "veneered" murine antigen-binding sites are thus designed to retain the murine CDR residues, the residues substantially adjacent to the CDRs, the residues identified as buried or mostly buried (solvent inaccessible), the residues believed to participate in non-covalent (*e.g.*, electrostatic and hydrophobic) contacts between heavy and light chain domains, and the residues from conserved structural regions of the FRs which are believed to influence the "canonical" tertiary structures of the CDR loops. These design criteria are then used to prepare recombinant

nucleotide sequences which combine the CDRs of both the heavy and light chain of a murine antigen-binding site into human-appearing FRs that can be used to transfect mammalian cells for the expression of recombinant human antibodies which exhibit the antigen specificity of the murine antibody molecule.

In another embodiment of the invention, monoclonal antibodies of the present invention may be coupled to one or more therapeutic agents. Suitable agents in this regard include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides include ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi. Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, and pokeweed antiviral protein.

A therapeutic agent may be coupled (*e.g.*, covalently bonded) to a suitable monoclonal antibody either directly or indirectly (*e.g.*, via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups,

sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, *e.g.*, U.S. Patent No. 4,671,958, to Rodwell *et al.*

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710, to Spitler), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014, to Senter *et al.*), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045, to Kohn *et al.*), by serum complement-mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958, to Rodwell *et al.*), and acid-catalyzed hydrolysis (*e.g.*, U.S. Patent No. 4,569,789, to Blattler *et al.*).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers that provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (*e.g.*, U.S. Patent No. 4,507,234, to Kato *et al.*), peptides and polysaccharides such as aminodextran (*e.g.*, U.S. Patent No. 4,699,784, to Shih *et al.*). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (*e.g.*, U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For

example, U.S. Patent No. 4,673,562, to Davison *et al.* discloses representative chelating compounds and their synthesis.

The above antibody types and procedures for making the same are provide for purposes of illustration but not by way of limitation. It will be understood that these and many other methodologies are known and established in the art for producing and characterizing antibodies and other binding agents, and these methodologies may be used in the context of the present invention.

Polypeptide Compositions

Another aspect of the present invention provides cancer-associated polypeptides. In certain related embodiments, the polypeptides are used, for example, in the context of pharmaceutical and/or vaccine compositions for the treatment of cancer.

As used herein, the term "polypeptide" " is used in its conventional meaning, *i.e.*, as a sequence of amino acids. The polypeptides are not limited to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide, and such terms may be used interchangeably herein unless specifically indicated otherwise. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. A polypeptide may be an entire protein, or a subsequence thereof. Particular polypeptides of interest in the context of this invention are amino acid subsequences comprising epitopes, *i.e.*, antigenic determinants substantially responsible for the immunogenic properties of a polypeptide and being capable of evoking an immune response.

In certain compositions and methods of the invention, preferred polypeptides comprise those set forth in any one of SEQ ID NOs: 1-24, or a variant or fragment of any of the foregoing, such as a variant or fragment having at least 70%, 80%, 90% or 95% identity thereto.

The polypeptides of the present invention are sometimes herein referred to as cancer-associated proteins or tumor polypeptides or oncofactor polypeptides, as an indication of their cancer association, such as their increased levels of expression and/or

activity in tumor samples. Thus, in certain embodiments, the terms "oncofactor," "tumor polypeptide," or "cancer-associated polypeptide," are generally used interchangeably and refer to a polypeptide sequence of the present invention, or a polynucleotide sequence encoding such a polypeptide, that is expressed and/or active in a substantial proportion of tumor samples, for example preferably greater than about 20%, more preferably greater than about 30%, and most preferably greater than about 50% or more of tumor samples tested, at a level that is at least two fold, and preferably at least five fold, greater than the level of expression in normal tissues, as determined using a representative assay provided herein. Oncofactor polypeptides of the invention having increased levels of expression and/or activity in tumor cells find particular utility both as a diagnostic marker as well as a therapeutic target, as further described below.

In certain embodiments, the polypeptides used according to the compositions and/or method of the present invention are immunogenic, *i.e.*, they react detectably within an immunoassay (such as an ELISA or T-cell stimulation assay) with antisera and/or T-cells from a patient with cancer. Screening for immunogenic activity can be performed using techniques well known to the skilled artisan. For example, such screens can be performed using methods such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In one illustrative example, a polypeptide may be immobilized on a solid support and contacted with patient sera to allow binding of antibodies within the sera to the immobilized polypeptide. Unbound sera may then be removed and bound antibodies detected using, for example, ^{125}I-labeled Protein A.

As would be recognized by the skilled artisan, immunogenic portions of the polypeptides disclosed herein are also encompassed by the present invention. An "immunogenic portion," as used herein, is a fragment of an immunogenic polypeptide of the invention that itself is immunologically reactive (*i.e.,* specifically binds) with the B-cells and/or T-cell surface antigen receptors that recognize the polypeptide. Immunogenic portions may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides for the ability to react with antigen-specific antibodies, antisera and/or T-cell lines or

clones. As used herein, antisera and antibodies are "antigen-specific" if they specifically bind to an antigen (*i.e.,* they react with the protein in an ELISA or other immunoassay, and do not react detectably with unrelated proteins). Such antisera and antibodies may be prepared as described herein, and using well-known techniques.

In one illustrative embodiment, an immunogenic portion of a polypeptide of the present invention is a portion that reacts with antisera and/or T-cells at a level that is not substantially less than the reactivity of the full-length polypeptide (*e.g.,* in an ELISA and/or T-cell reactivity assay). Preferably, the level of immunogenic activity of the immunogenic portion is at least about 50%, preferably at least about 70% and most preferably greater than about 90% of the immunogenicity for the full-length polypeptide. In some instances, preferred immunogenic portions will be identified that have a level of immunogenic activity greater than that of the corresponding full-length polypeptide, *e.g.*, having greater than about 100% or 150% or more immunogenic activity.

The present invention, in other embodiments, employs polypeptide fragments comprising at least about 5, 10, 15, 20, 25, 50, or 100 contiguous amino acids, or more, including all intermediate lengths, of a polypeptide compositions set forth herein, such as those set forth in SEQ ID NOs: 1-24.

In still other embodiments, the present invention employs variants of the polypeptide compositions described herein in the compositions and methods described herein. Polypeptide variants generally encompassed by the present invention will typically exhibit at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity (determined as described below), along its length, to a polypeptide sequences set forth herein.

A polypeptide "variant," as the term is used herein, is a polypeptide that typically differs from a polypeptide specifically disclosed herein in one or more substitutions, deletions, additions and/or insertions. Such variants may be naturally occurring or may be synthetically generated, for example, by modifying one or more of the above polypeptide sequences of the invention and evaluating their immunogenic activity as described herein and/or using any of a number of techniques well known in the art.

In many instances, a variant will contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. As described above, modifications may be made in the structure of the polynucleotides and polypeptides of the present invention and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics, *e.g.*, with immunogenic characteristics. When it is desired to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, immunogenic variant or portion of a polypeptide of the invention, one skilled in the art will typically change one or more of the codons of the encoding DNA sequence according to Table 1.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

Table 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, 1982). These values are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine

(+2.5); methionine (+1.9); alanine (+1.8); glycine (–0.4); threonine (–0.7); serine (–0.8); tryptophan (–0.9); tyrosine (–1.3); proline (–1.6); histidine (–3.2); glutamate (–3.5); glutamine (–3.5); aspartate (–3.5); asparagine (–3.5); lysine (–3.9); and arginine (–4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101 (specifically incorporated herein by reference in its entirety), states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (–1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (–2.3); phenylalanine (–2.5); tryptophan (–3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those

of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition, any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl-methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Amino acid substitutions may further be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. In a preferred embodiment, variant polypeptides differ from a native sequence by substitution, deletion or addition of five amino acids or fewer. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydropathic nature of the polypeptide.

Polypeptides may comprise a signal (or leader) sequence at the N-terminal end of the protein, which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.,* poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

When comparing polypeptide sequences, two sequences are said to be "identical" if the sequence of amino acids in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O., (1978) *A model of evolutionary change in proteins – Matrices for detecting distant relationships*. In Dayhoff, M.O. (ed.) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) *Unified Approach to Alignment and Phylogenes*, pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M., *CABIOS 5*:151-153 (1989); Myers, E.W. and Muller W., *CABIOS 4*:11-17 (1988); Robinson, E.D., *Comb. Theor 11*:105 (1971); Saitou, N. Nei, M., *Mol. Biol. Evol. 4*:406-425 (1987); Sneath, P.H.A. and Sokal, R.R., *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA (1973); Wilbur, W.J. and Lipman, D.J., *Proc. Natl. Acad., Sci. USA 80*:726-730 (1983).

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman, *Add. APL. Math* 2:482 (1981), by the identity alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity methods of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nucl. Acids Res.* 25:3389-3402 (1977), and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides and polypeptides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. For amino acid sequences, a scoring matrix can be used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment.

In one preferred approach, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.,* gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.,* the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

Fusion Polypeptides

Within other illustrative embodiments, an oncofactor polypeptide of the invention may be in the form of a fusion polypeptide that comprises multiple cancer-associated polypeptides as described herein, or that comprises at least one cancer-associated polypeptide as described herein and an unrelated sequence, such as a known tumor protein or other heterologous sequence of interest. A fusion partner may, for

example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the polypeptide or to enable the polypeptide to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the polypeptide.

Fusion polypeptides may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion polypeptide is expressed as a recombinant polypeptide, allowing the production of increased levels, relative to a non-fused polypeptide, in an expression system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion polypeptide that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion polypeptide using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea *et al.*, *Gene 40*:39-46, 1985; Murphy *et al.*, *Proc. Natl. Acad. Sci. USA 83*:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S.

Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons required to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

Cancer-associated polypeptides (*e.g.,* oncofactors) of the invention are prepared using any of a variety of well known synthetic and/or recombinant techniques, the latter of which are further described below. Polypeptides, portions and other variants generally less than about 150 amino acids can be generated by synthetic means, using techniques well known to those of ordinary skill in the art. In one illustrative example, such polypeptides are synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem. Soc. 85*:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer/Applied BioSystems Division (Foster City, CA), and may be operated according to the manufacturer's instructions.

In general, polypeptide compositions (including fusion polypeptides) of the invention are isolated. An "isolated" polypeptide is one that is removed from its original environment. For example, a naturally-occurring protein or polypeptide is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are also purified, *e.g.*, are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure.

Polynucleotide Compositions

As noted above, another aspect of the invention relates to the polynucleotides encoding the oncofactor polypeptides described herein, and their use in the context of pharmaceutical and diagnostic compositions for the treatment and detection of cancer.

The terms "DNA" and "polynucleotide" are used essentially interchangeably herein to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. "Isolated," as used herein, means that a polynucleotide is substantially away from other coding sequences, and that the DNA molecule does not contain large portions of unrelated coding DNA, such as large chromosomal fragments or other functional genes or polypeptide coding regions. Of course, this refers to the DNA molecule as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

As will be understood by those skilled in the art, the polynucleotide compositions of this invention can include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, peptides and the like. Such segments may be naturally isolated, or modified synthetically by the hand of man.

As will be also recognized by the skilled artisan, polynucleotides of the invention may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules may include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Polynucleotides may comprise a native sequence (*i.e.,* an endogenous sequence that encodes a polypeptide/protein of the invention or a portion thereof) or may comprise a sequence that encodes a variant or derivative, preferably and immunogenic variant or derivative, of such a sequence.

In certain embodiments, the present invention employs polynucleotide variants having substantial identity to a sequence encoding a cancer-assocaited polypeptide sequences disclosed herein in SEQ ID NOs: 1-24, for example those comprising at least 70% sequence identity, preferably at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or higher, sequence identity compared to a polynucleotide sequence of this invention using the methods described herein, (*e.g.*, BLAST analysis using standard parameters, as described below). One skilled in this art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like.

Typically, polynucleotide variants will contain one or more substitutions, additions, deletions and/or insertions, preferably such that the immunogenicity of the polypeptide encoded by the variant polynucleotide is not substantially diminished relative to a polypeptide encoded by a polynucleotide sequence specifically set forth herein). The term "variants" should also be understood to encompass homologous genes of xenogenic origin.

In additional embodiments, the present invention provides polynucleotide fragments comprising or consisting of various lengths of contiguous stretches of sequence identical to or complementary to one or more of the sequences described herein. For example, polynucleotides are provided by this invention that comprise or consist of at least about 10, 15, 20, 30, 40, 50, 75, 100, 150, 200, 300, 400, 500 or 1000 or more contiguous nucleotides of a sequence encoding a polypeptide disclosed herein as well as all intermediate lengths there between. It will be readily understood that "intermediate lengths", in this context, means any length between the quoted values, such as 16, 17, 18, 19, *etc.*; 21, 22, 23, *etc.*; 30, 31, 32, *etc.*; 50, 51, 52, 53, *etc.*; 100, 101, 102, 103, *etc.*; 150, 151, 152, 153, *etc.*; including all integers through 200-500; 500-1,000, and the like. A polynucleotide sequence as described here may be extended at one or both ends by additional nucleotides not found in the native sequence. This additional sequence may consist of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,

16, 17, 18, 19, or 20 nucleotides at either end of the disclosed sequence or at both ends of the disclosed sequence.

In another embodiment of the invention, polynucleotide compositions are provided that are capable of hybridizing under moderate to high stringency conditions to a polynucleotide sequence that encodes a polypeptide sequence provided herein, or a fragment thereof, or a complementary sequence thereof. Hybridization techniques are well known in the art of molecular biology. For purposes of illustration, suitable moderately stringent conditions for testing the hybridization of a polynucleotide of this invention with other polynucleotides include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-60°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS. One skilled in the art will understand that the stringency of hybridization can be readily manipulated, such as by altering the salt content of the hybridization solution and/or the temperature at which the hybridization is performed. For example, in another embodiment, suitable highly stringent hybridization conditions include those described above, with the exception that the temperature of hybridization is increased, *e.g.*, to 60-65°C or 65-70°C.

The polynucleotides of the present invention, or fragments thereof, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, illustrative polynucleotide segments with total lengths of about 10,000, about 5000, about 3000, about 2,000, about 1,000, about 500, about 200, about 100, about 50 base pairs in length, and the like, (including all intermediate lengths) are contemplated to be useful in many implementations of this invention.

When comparing polynucleotide sequences, two sequences are said to be "identical" if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two

sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J., *Unified Approach to Alignment and Phylogenes*, pp. 626-645 (1990); *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M., *CABIOS 5*:151-153 (1989); Myers, E.W. and Muller W., *CABIOS 4*:11-17 (1988); Robinson, E.D., *Comb. Theor 11*:105 (1971); Santou, N. Nes, M., *Mol. Biol. Evol. 4*:406-425 (1987); Sneath, P.H.A. and Sokal, R.R., *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA (1973); Wilbur, W.J. and Lipman, D.J., *Proc. Natl. Acad., Sci. USA 80*:726-730 (1983).

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman, *Add. APL. Math* 2:482 (1981), by the identity alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity methods of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nucl. Acids Res.* 25:3389-3402

(1977), and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. In one illustrative example, cumulative scores can be calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments, (B) of 50, expectation (E) of 10, M=5, N=-4 and a comparison of both strands.

In certain embodiments, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.,* gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.,* the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides

that vary due to differences in codon usage are specifically contemplated by the present invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the present invention. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

Therefore, in another embodiment of the invention, a mutagenesis approach, such as site-specific mutagenesis, is employed for the preparation of variants and/or derivatives of the polypeptides described herein. By this approach, specific modifications in a polypeptide sequence can be made through mutagenesis of the underlying polynucleotides that encode them. These techniques provides a straightforward approach to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the polynucleotide.

Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or otherwise change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments of the present invention, the inventors contemplate the mutagenesis of the disclosed polynucleotide sequences to alter one or more properties of the encoded polypeptide, such as the immunogenicity of a polypeptide vaccine. The techniques of site-specific mutagenesis are well-known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site-specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25

nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy *et al.*, 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis *et al.*, 1982, each incorporated herein by reference, for that purpose.

In other embodiments of the present invention, the polynucleotide sequences provided herein can be advantageously used as probes or primers for nucleic acid hybridization, *e.g.*, for use in diagnosis and/or monitoring of cancer in a subject. As such, it is contemplated that nucleic acid segments that comprise or consist of a sequence region of at least about a 15 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 15 nucleotide long contiguous sequence described herein will find particular utility. Longer contiguous identical or complementary sequences, *e.g.*, those of about 20, 30, 40, 50, 100, 200, 500, 1000 (including all intermediate lengths) and even up to full length sequences will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to a sequence of interest will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are also envisioned, such as the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Polynucleotide molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so (including intermediate lengths as well), identical or complementary to a polynucleotide sequence described herein, are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. This would allow

a gene product, or fragment thereof, to be analyzed, both in diverse cell types and also in various bacterial cells. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 15 and about 100 nucleotides, but larger contiguous complementarity stretches may be used, according to the length complementary sequences one wishes to detect.

The use of a hybridization probe of about 15-25 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 15 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 25 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences described herein. All that is required is to review the sequences from about 15-25 nucleotides in length up to and including the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and primer sequences may be governed by various factors that are known and established in the art.

Small polynucleotide segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U. S. Patent 4,683,202 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

The nucleotide sequences according to the invention may be used for their ability to selectively form duplex molecules with complementary stretches of the

entire gene or gene fragments of interest. Depending on the application envisioned, one will typically desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by a salt concentration of from about 0.02 M to about 0.15 M salt at temperatures of from about 50°C to about 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating related sequences.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template, less stringent (reduced stringency) hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ salt conditions such as those of from about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

According to another embodiment of the present invention, polynucleotide compositions comprising antisense oligonucleotides are provided for use in the compositions and methods herein. Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, provide a therapeutic approach by which a disease can be treated by inhibiting the synthesis of proteins that contribute to the disease. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829). Further, examples of

antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal $GABA_A$ receptor and human EGF (Jaskulski *et al.*, *Science* 1988 Jun 10;240(4858):1544-6; Vasanthakumar and Ahmed, Cancer Commun. 1989;1(4):225-32; Peris *et al.*, *Brain Res Mol Brain Res*. 1998 Jun 15;57(2):310-20; U. S. Patent 5,801,154; U.S. Patent 5,789,573; U. S. Patent 5,718,709 and U.S. Patent 5,610,288). Antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.,* cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683).

Therefore, in certain embodiments, the present invention provides oligonucleotide sequences that comprise all, or a portion of, any sequence that is capable of specifically binding to polynucleotide sequence described herein, or a complement thereof. In one embodiment, the antisense oligonucleotides comprise DNA or derivatives thereof. In another embodiment, the oligonucleotides comprise RNA or derivatives thereof. In another embodiment, the oligonucleotides are modified DNAs comprising a phosphorothioated modified backbone. In another embodiment, the oligonucleotide sequences comprise peptide nucleic acids or derivatives thereof. In each case, illustrative compositions comprise a sequence region that is complementary, and more preferably substantially-complementary, and even more preferably, completely complementary to one or more portions of polynucleotides described herein. Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense compositions may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA, are those which are at or near the AUG translation initiation codon, and those sequences which are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.,* Nucleic Acids Res. 1997, 25(17):3389-402).

According to another embodiment of the invention, the polynucleotide compositions described herein are used in the design and preparation of ribozyme molecules for use in compositions and methods for inhibiting the expression of the tumor polypeptides and proteins of the present invention in tumor cells. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, *Proc. Natl. Acad. Sci. USA*. 1987 Dec;84(24):8788-92; Forster and Symons, Cell. 1987 Apr 24;49(2):211-20). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, Cell. 1981 Dec;27(3 Pt 2):487-96; Michel and Westhof, J Mol Biol. 1990 Dec 5;216(3):585-610; Reinhold-Hurek and Shub, Nature. 1992 May 14;357(6374):173-6). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Enzymatic RNAs can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act

enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, *Proc. Natl. Acad. Sci. USA*. 1992 Aug 15;89(16):7305-9). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* Nucleic Acids Res. 1992 Sep 11;20(17):4559-65. Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz, Biochemistry 1989 Jun 13;28(12):4929-33; Hampel *et al.,* Nucleic Acids Res. 1990 Jan 25;18(2):299-304 and U. S. Patent 5,631,359. An example of the hepatitis δ virus motif is described by Perrotta and Been, Biochemistry. 1992 Dec 1;31(47):11843-52; an example of the RNaseP motif is described by Guerrier-Takada *et al.*, Cell. 1983 Dec;35(3 Pt 2):849-57; Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, Cell. 1990 May 18;61(4):685-96; Saville and Collins, *Proc. Natl. Acad. Sci. USA*, 88(19):8826-30 (Oct 1 1991); Collins and Olive, *Biochemistry 32*(11):2795-9 (Mar 23 1993); and an example of the Group I intron is described in (U. S. Patent 4,987,071). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595, each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as

described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

In another embodiment of the invention, peptide nucleic acids (PNAs) compositions are provided. PNA is a DNA mimic in which the nucleobases are attached to a pseudopeptide backbone (Good and Nielsen, Antisense Nucleic Acid Drug Dev. 1997 7(4) 431-37). PNA is able to be utilized in a number methods that traditionally have used RNA or DNA. Often PNA sequences perform better in techniques than the corresponding RNA or DNA sequences and have utilities that are not inherent to RNA or DNA. A review of PNA including methods of making, characteristics of, and methods of using, is provided by Corey (*Trends Biotechnol 15*(6):224-9 (Jun 1997)). As such, in certain embodiments, one may prepare PNA sequences that are complementary to one or more portions of the ACE mRNA sequence, and such PNA compositions may be used to regulate, alter, decrease, or reduce the translation of ACE-specific mRNA, and thereby alter the level of ACE activity in a host cell to which such PNA compositions have been administered.

PNAs have 2-aminoethyl-glycine linkages replacing the normal phosphodiester backbone of DNA (Nielsen *et al.*, *Science 254*(5037):1497-500 (Dec 6 1991); Hanvey *et al.*, *Science 258*(5087):1481-5 (Nov 27 1992); Hyrup and Nielsen, *Bioorg. Med. Chem. 4*(1):5-23 (Jan 1996). This chemistry has three important consequences: firstly, in contrast to DNA or phosphorothioate oligonucleotides, PNAs are neutral molecules; secondly, PNAs are achiral, which avoids the need to develop a stereoselective synthesis; and thirdly, PNA synthesis uses standard Boc or Fmoc protocols for solid-phase peptide synthesis, although other methods, including a modified Merrifield method, have been used.

PNA monomers or ready-made oligomers are commercially available from PerSeptive Biosystems (Framingham, MA). PNA syntheses by either Boc or Fmoc protocols are straightforward using manual or automated protocols (Norton *et al.*, *Bioorg. Med. Chem. 3*(4):437-45 (Apr 1995)). The manual protocol lends itself to the production of chemically modified PNAs or the simultaneous synthesis of families of closely related PNAs.

As with peptide synthesis, the success of a particular PNA synthesis will depend on the properties of the chosen sequence. For example, while in theory PNAs can incorporate any combination of nucleotide bases, the presence of adjacent purines can lead to deletions of one or more residues in the product. In expectation of this difficulty, it is suggested that, in producing PNAs with adjacent purines, one should repeat the coupling of residues likely to be added inefficiently. This should be followed by the purification of PNAs by reverse-phase high-pressure liquid chromatography, providing yields and purity of product similar to those observed during the synthesis of peptides.

Modifications of PNAs for a given application may be accomplished by coupling amino acids during solid-phase synthesis or by attaching compounds that contain a carboxylic acid group to the exposed N-terminal amine. Alternatively, PNAs can be modified after synthesis by coupling to an introduced lysine or cysteine. The ease with which PNAs can be modified facilitates optimization for better solubility or for specific functional requirements. Once synthesized, the identity of PNAs and their derivatives can be confirmed by mass spectrometry. Several studies have made and utilized modifications of PNAs (for example, Norton *et al.*, *Bioorg Med Chem 3*(4):437-45 (Apr 1995); Petersen *et al.*, *J Pept Sci 1*(3):175-83 (May-Jun 1995); Orum *et al.*, *Biotechniques 19*(3):472-80 (Sep 1995); Footer *et al.*, *Biochemistry*. 1996 Aug 20;35(33):10673-9; Griffith *et al.*, *Nucleic Acids Res 23*(15):3003-8 (Aug 11 1995); Pardridge *et al.*, *Proc. Natl. Acad. Sci. USA*. *92*(12):5592-6 (Jun 6 1995); Boffa *et al.*, *Proc. Natl. Acad. Sci. USA*. *92*(6):1901-5 (Mar 14 1995); Gambacorti-Passerini *et al.*, *Blood 88*(4):1411-7 (Aug 15 1996); Armitage *et al., Proc. Natl. Acad. Sci. USA*. *94*(23):12320-5 (Nov 11 1997); Seeger *et al., Biotechniques 23*(3):512-7 (Sep 1997)). U.S. Patent No. 5,700,922 discusses PNA-DNA-PNA chimeric molecules and their uses in diagnostics, modulating protein in organisms, and treatment of conditions susceptible to therapeutics.

Methods of characterizing the antisense binding properties of PNAs are discussed in Rose (*Anal Chem 65*(24):3545-9 (Dec 15 1993) and Jensen *et al.* (Biochemistry. 1997 Apr 22;36(16):5072-7). Rose uses capillary gel electrophoresis to determine binding of PNAs to their complementary oligonucleotide, measuring the

relative binding kinetics and stoichiometry. Similar types of measurements were made by Jensen *et al.* using BIAcore™ technology.

Other applications of PNAs that have been described and will be apparent to the skilled artisan include use in DNA strand invasion, antisense inhibition, mutational analysis, enhancers of transcription, nucleic acid purification, isolation of transcriptionally active genes, blocking of transcription factor binding, genome cleavage, biosensors, *in situ* hybridization, and the like.

Polynucleotide Identification, Characterization and Expression

Polynucleotides (and polypeptide) compositions of the present invention may be identified, prepared and/or manipulated using any of a variety of well established techniques (see generally, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989, and other like references).

Many template dependent processes are available to amplify a target sequence of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR™) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in its entirety. Briefly, in PCR™, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase (*e.g.*, *Taq* polymerase). If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the target to form reaction products, excess primers will bind to the target and to the reaction product and the process is repeated. Preferably reverse transcription and PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art.

Any of a number of other template dependent processes, many of which are variations of the PCR™amplification technique, are readily known and available in

the art. Illustratively, some such methods include the ligase chain reaction (referred to as LCR), described, for example, in Eur. Pat. Appl. Publ. No. 320,308 and U.S. Patent No. 4,883,750; Qbeta Replicase, described in PCT Intl. Pat. Appl. Publ. No. PCT/US87/00880; Strand Displacement Amplification (SDA) and Repair Chain Reaction (RCR). Still other amplification methods are described in Great Britain Pat. Appl. No. 2 202 328, and in PCT Intl. Pat. Appl. Publ. No. PCT/US89/01025. Other nucleic acid amplification procedures include transcription-based amplification systems (TAS) (PCT Intl. Pat. Appl. Publ. No. WO 88/10315), including nucleic acid sequence based amplification (NASBA) and 3SR. Eur. Pat. Appl. Publ. No. 329,822 describes a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA). PCT Intl. Pat. Appl. Publ. No. WO 89/06700 describes a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. Other amplification methods such as "RACE" (Frohman, 1990), and "one-sided PCR" (Ohara, 1989) are also well-known to those of skill in the art.

An amplified portion of a polynucleotide of the present invention may be used to isolate a full length gene from a suitable library (*e.g.,* a tumor cDNA library) using well known techniques. Within such techniques, a library (cDNA or genomic) is screened using one or more polynucleotide probes or primers suitable for amplification. Preferably, a library is size-selected to include larger molecules. Random primed libraries may also be preferred for identifying 5' and upstream regions of genes. Genomic libraries are preferred for obtaining introns and extending 5' sequences.

For hybridization techniques, a partial sequence may be labeled (*e.g.,* by nick-translation or end-labeling with ^{32}P) using well known techniques. A bacterial or bacteriophage library is then generally screened by hybridizing filters containing denatured bacterial colonies (or lawns containing phage plaques) with the labeled probe (*see* Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989). Hybridizing colonies or plaques are selected and expanded, and the DNA is isolated for further analysis. cDNA clones may be analyzed to determine the amount of additional sequence by, for example, PCR

using a primer from the partial sequence and a primer from the vector. Restriction maps and partial sequences may be generated to identify one or more overlapping clones. The complete sequence may then be determined using standard techniques, which may involve generating a series of deletion clones. The resulting overlapping sequences can then assembled into a single contiguous sequence. A full length cDNA molecule can be generated by ligating suitable fragments, using well known techniques.

As will be understood by those of skill in the art, it may be advantageous in some instances to produce polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce a recombinant RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

Moreover, the polynucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter polypeptide encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the gene product. For example, DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. In addition, site-directed mutagenesis may be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, or introduce mutations, and so forth.

Sequences encoding a desired polypeptide may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers, M. H. *et al.* (1980) *Nucl. Acids Res. Symp. Ser.* 215-223, Horn, T. *et al.* (1980) *Nucl. Acids Res. Symp. Ser.* 225-232). Alternatively, the protein itself may be produced using chemical methods to synthesize the amino acid sequence of a polypeptide, or a portion thereof. For example, peptide synthesis can be performed using various solid-phase techniques (Roberge, J. Y. *et al.* (1995) *Science 269*:202-204) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer, Palo Alto, CA).

A newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography (*e.g.*, Creighton, T. (1983) Proteins, Structures and Molecular Principles, WH Freeman and Co., New York, N.Y.) or other comparable techniques available in the art. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure). Additionally, the amino acid sequence of a polypeptide, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

In order to express a desired polypeptide, the nucleotide sequences encoding the polypeptide, or functional equivalents, may be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding a polypeptide of interest and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook, J. *et al.* (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., and Ausubel, F. M. *et al.* (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York. N.Y.

A variety of expression vector/host systems may be utilized to contain and express polynucleotide sequences. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (*e.g.*, baculovirus); plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids); or animal cell systems.

The "control elements" or "regulatory sequences" present in an expression vector are those non-translated regions of the vector—enhancers, promoters,

5' and 3' untranslated regions—which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the pBLUESCRIPT phagemid (Stratagene, La Jolla, Calif.) or pSPORT1 plasmid (Gibco BRL, Gaithersburg, MD) and the like may be used. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are generally preferred. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding a polypeptide, vectors based on SV40 or EBV may be advantageously used with an appropriate selectable marker.

In mammalian host cells, a number of viral-based expression systems are generally available. For example, in cases where an adenovirus is used as an expression vector, sequences encoding a polypeptide of interest may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the polypeptide in infected host cells (Logan, J. and Shenk, T. (1984) *Proc. Natl. Acad. Sci. 81*:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding a polypeptide of interest. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding the polypeptide, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are

appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. *et al.* (1994) *Results Probl. Cell Differ. 20*:125-162).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation. glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells such as CHO, COS, HeLa, MDCK, HEK293, and WI38, which have specific cellular machinery and characteristic mechanisms for such post-translational activities, may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is generally preferred. For example, cell lines which stably express a polynucleotide of interest may be transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. *et al.* (1977) *Cell 11*:223-32) and adenine phosphoribosyltransferase (Lowy, I. *et al.* (1990) *Cell 22*:817-23) genes which can be employed in tk.sup.- or aprt.sup.- cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. *et al.* (1980) *Proc. Natl. Acad. Sci. 77*:3567-70); npt, which confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin, F. et al (1981) *J. Mol. Biol. 150*:1-14); and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, *supra*).

Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S. C. and R. C. Mulligan (1988) *Proc. Natl. Acad. Sci. 85*:8047-51). The use of visible markers has gained popularity with such markers as anthocyanins, beta-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C. A. *et al.* (1995) *Methods Mol. Biol. 55*:121-131).

Alternatively, host cells that contain and express a desired polynucleotide sequence may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include, for example, membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein.

A variety of protocols for detecting and measuring the expression of polynucleotide-encoded products, using either polyclonal or monoclonal antibodies specific for the product are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on a given polypeptide may be preferred for some applications, but a competitive binding assay may also be employed. These and other assays are described, among other places, in Hampton, R. *et al.* (1990; Serological Methods, a Laboratory Manual, APS Press, St Paul. Minn.) and Maddox, D. E. *et al.* (1983; *J. Exp. Med. 158*:1211-1216).

A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the sequences, or any portions thereof may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA

probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits. Suitable reporter molecules or labels, which may be used include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with a polynucleotide sequence of interest may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a recombinant cell may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides of the invention may be designed to contain signal sequences which direct secretion of the encoded polypeptide through a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may be used to join sequences encoding a polypeptide of interest to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). The inclusion of cleavable linker sequences such as those specific for Factor XA or enterokinase (Invitrogen. San Diego, Calif.) between the purification domain and the encoded polypeptide may be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing a polypeptide of interest and a nucleic acid encoding 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography) as described in Porath, J. *et al.* (1992, *Prot. Exp. Purif. 3*:263-281) while the enterokinase cleavage site provides a means for purifying the desired polypeptide from the fusion protein. A discussion of vectors which contain fusion proteins is provided in Kroll, D. J. *et al.* (1993; *DNA Cell Biol. 12*:441-453).

In addition to recombinant production methods, polypeptides of the invention, and fragments thereof, may be produced by direct peptide synthesis using

solid-phase techniques (Merrifield J. (1963) *J. Am. Chem. Soc. 85*:2149-2154). Protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Alternatively, various fragments may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

T Cell Compositions

The present invention, in another aspect, provides T cells specific for a cancer-associated polypeptide disclosed herein (*e.g.,* oncofactor), or for a variant or derivative or fragment thereof. Such cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be isolated from bone marrow, peripheral blood, or a fraction of bone marrow or peripheral blood of a patient, using a commercially available cell separation system, such as the Isolex™ System, available from Nexell Therapeutics, Inc. (Irvine, CA; see also U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may be derived from related or unrelated humans, non-human mammals, cell lines or cultures.

T cells may be stimulated with a cancer-associated polypeptide, polynucleotide encoding a polypeptide and/or an antigen presenting cell (APC) that expresses such a polypeptide. Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the polypeptide of interest. Preferably, a tumor polypeptide or polynucleotide of the invention is present within a delivery vehicle, such as a microsphere, to facilitate the generation of specific T cells.

T cells are considered to be specific for a polypeptide of the present invention if the T cells specifically proliferate, secrete cytokines or kill target cells coated with the polypeptide or expressing a gene encoding the polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a chromium release assay or proliferation assay, a stimulation index of more than two fold increase in lysis and/or proliferation, compared to negative controls,

indicates T cell specificity. Such assays may be performed, for example, as described in Chen *et al.*, *Cancer Res. 54*:1065-1070, 1994. Alternatively, detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (*e.g.,* by pulse-labeling cultures of T cells with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Contact with a tumor polypeptide (100 ng/ml–100 μg/ml, preferably 200 ng/ml–25 μg/ml) for 3-7 days will typically result in at least a two fold increase in proliferation of the T cells. Contact as described above for 2-3 hours should result in activation of the T cells, as measured using standard cytokine assays in which a two fold increase in the level of cytokine release (*e.g.,* TNF or IFN-γ) is indicative of T cell activation (*see* Coligan *et al.*, Current Protocols in Immunology, vol. 1, Wiley Interscience (Greene 1998)). T cells that have been activated in response to a tumor polypeptide, polynucleotide or polypeptide-expressing APC may be $CD4^+$ and/or $CD8^+$. Tumor polypeptide-specific T cells may be expanded using standard techniques. Within certain embodiments, the T cells are derived from a patient, a related donor or an unrelated donor, and are administered to the patient following stimulation and expansion.

For therapeutic purposes, $CD4^+$ or $CD8^+$ T cells that proliferate in response to a tumor polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways. For example, the T cells can be re-exposed to a tumor polypeptide, or a short peptide corresponding to an immunogenic portion of such a polypeptide, with or without the addition of T cell growth factors, such as interleukin-2, and/or stimulator cells that synthesize a tumor polypeptide. Alternatively, one or more T cells that proliferate in the presence of the tumor polypeptide can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution.

The T cell receptor (TCR) consists of 2 different, highly variable polypeptide chains, termed the T-cell receptor α and β chains, that are linked by a disulfide bond (Janeway, Travers, Walport. Immunobiology. Fourth Ed., 148-159. Elsevier Science Ltd/Garland Publishing. 1999). The α/β heterodimer complexes with

the invariant CD3 chains at the cell membrane. This complex recognizes specific antigenic peptides bound to MHC molecules. The enormous diversity of TCR specificities is generated much like immunoglobulin diversity, through somatic gene rearrangement. The β chain genes contain over 50 variable (V), 2 diversity (D), over 10 joining (J) segments, and 2 constant region segments (C). The α chain genes contain over 70 V segments, and over 60 J segments but no D segments, as well as one C segment. During T cell development in the thymus, the D to J gene rearrangement of the β chain occurs, followed by the V gene segment rearrangement to the DJ. This functional VDJβ exon is transcribed and spliced to join to a Cβ. For the α chain, a Vα gene segment rearranges to a Jα gene segment to create the functional exon that is then transcribed and spliced to the Cα. Diversity is further increased during the recombination process by the random addition of P and N-nucleotides between the V, D, and J segments of the b chain and between the V and J segments in the α chain (Janeway, Travers, Walport. Immunobiology. Fourth Ed., 98 and 150. Elsevier Science Ltd/Garland Publishing. 1999).

The present invention, in another aspect, provides TCRs specific for a cancer-associated polypeptide disclosed herein, or for a variant or derivative thereof. In accordance with the present invention, polynucleotide and amino acid sequences are provided for the V-J or V-D-J junctional regions or parts thereof for the alpha and beta chains of the T-cell receptor which recognize tumor polypeptides described herein. In general, this aspect of the invention relates to T-cell receptors which recognize or bind tumor polypeptides presented in the context of MHC. In a preferred embodiment the tumor antigens recognized by the T-cell receptors comprise a polypeptide of the present invention. For example, cDNA encoding a TCR specific for a tumor peptide can be isolated from T cells specific for a tumor polypeptide using standard molecular biological and recombinant DNA techniques.

This invention further includes the T-cell receptors or analogs thereof having substantially the same function or activity as the T-cell receptors of this invention which recognize or bind tumor polypeptides. Such receptors include, but are not limited to, a fragment of the receptor, or a substitution, addition or deletion mutant of a T-cell receptor provided herein. This invention also encompasses polypeptides or

peptides that are substantially homologous to the T-cell receptors provided herein or that retain substantially the same activity. The term "analog" includes any protein or polypeptide having an amino acid residue sequence substantially identical to the T-cell receptors provided herein in which one or more residues, preferably no more than 5 residues, more preferably no more than 25 residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of the T-cell receptor as described herein.

The present invention further provides for suitable mammalian host cells, for example, non-specific T-cells, that are transfected with a polynucleotide encoding TCRs specific for a polypeptide described herein, thereby rendering the host cell specific for the polypeptide. The α and β chains of the TCR may be contained on separate expression vectors or alternatively, on a single expression vector that also contains an internal ribosome entry site (IRES) for cap-independent translation of the gene downstream of the IRES. Said host cells expressing TCRs specific for the polypeptide may be used, for example, for adoptive immunotherapy of cancer as discussed further below.

In further aspects of the present invention, cloned TCRs specific for a polypeptide recited herein may be used in a kit for the diagnosis of cancer. For example, the nucleic acid sequence or portions thereof, of tumor-specific TCRs can be used as probes or primers for the detection of expression of the rearranged genes encoding the specific TCR in a biological sample. In this respect, the present invention further provides for an assay for detecting messenger RNA or DNA encoding the TCR specific for a polypeptide.

Pharmaceutical & Other Compositions

Pharmaceutical compositions of the invention generally comprise one or more of the cancer-associated antibodies, polynucleotides, polypeptides, T-cells, or TCR compositions disclosed herein in pharmaceutically-acceptable carriers for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy.

It will be understood that, if desired, a composition as disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, other

proteins or polypeptides or various pharmaceutically-active agents. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA or DNA compositions.

Therefore, in another aspect of the present invention, pharmaceutical compositions are provided comprising one or more of the polynucleotide, polypeptide, antibody, TCR, and/or T-cell compositions described herein in combination with a physiologically acceptable carrier.

In one embodiment, the invention contemplates, in part, compositions comprising one or more antibodies to a cancer-associated polypeptide or oncofactor disclosed herein. In particular embodiments of the invention, the composition comprises one or more purified oncofactor antibodies and the composition is substantially free from endotoxin, has little or no aggregate formation, and the purified isolated polypeptide of the composition is soluble in a therapeutically acceptable formulation.

In one embodiment, the invention contemplates compositions comprising at least fragments thereof having at least one purified isolated antibody to an oncofactor wherein the antibody and composition are substantially free from endotoxin, wherein the p antibody has little or no aggregate formation, wherein the antibody is soluble in a therapeutically acceptable formulation and wherein the composition is substantially free of mammalian proinflammatory agents.

Endotoxins are toxins associated with certain bacteria, typically gram-negative bacteria, although endotoxins may be found in gram-positive bacteria, such as *Listeria monocytogenes*. The most prevalent endotoxins are lipopolysaccharides (LPS) or lipooligosaccharides (LOS) found in the outer membrane of various Gram-negative bacteria, and which represent a central pathogenic feature in the ability of these bacteria to cause disease. Small amounts of endotoxin in humans can produce fever, a lowering

of the blood pressure, and activation of inflammation and coagulation, among other adverse physiological effects. Therefore, it is often desirable to remove most or all traces of endotoxin from drugs and drug product containers, because even small amounts may cause adverse effects in humans. Producing formulations that are endotoxin-free can require special equipment, expert artisans, and can be significantly more expensive than making formulations that are not endotoxin-free.

Endotoxins can be detected using routine techniques known in the art. For example, the Limulus Ameobocyte Lysate assay, which utilizes blood from the horseshoe crab, is a very sensitive assay for detecting presence of endotoxin. In this test, very low levels of LPS can cause detectable coagulation of the limulus lysate due a powerful enzymatic cascade that amplifies this reaction. Endotoxins can also be quantitated by enzyme-linked immunosorbent assay (ELISA). As used herein, the term "endotoxin free" refers to compositions that contain at most trace amounts (*i.e.*, amounts having no adverse physiological effects to a subject) of endotoxin, and preferably undetectable amounts of endotoxin. In one embodiment, the term "endotoxin free" refers to a composition that is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% endotoxin free. In one embodiment, the term "endotoxin free" refers to endotoxin levels or an endotoxin profile that may be less than about 0.001, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08. 0.09, 0.1, 0.5, 1.0, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, or 10 endotoxin units (EU)/ml or EU/mg. Typically, 1 ng lipopolysaccharide (LPS) corresponds to about 1-10 EU.

To be substantially endotoxin free, endotoxin levels or endotoxin profile may be less than about 0.001, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08. 0.09, 0.1, 0.5, 1.0, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, or 10 EU/ml.

In certain embodiments, the invention contemplates, in part, an oncofactor antibody comprising an endotoxin profile of less than about 50 EU/mg, less than about 30 EU/mg , less than about 25 EU/mg , less than about 20 EU/mg , less than about 15 EU/mg , less than about 10 EU/mg , less than about 8 EU/mg , less than about 7 EU/mg, less than about 6 EU/mg , less than about 5 EU/mg, less than about 4 EU/mg, less than about 3 EU/mg less than about 2 EU/mg, less than about 1.5 EU/mg, less than about 1.4 EU/mg, less than about 1.3 EU/mg, less than about 1.2 EU/mg, less than

about 1.1 EU/mg, less than about 1.0 EU/mg, less than about 0.9 EU/mg, less than about 0.8 EU/mg, less than about 0.7 EU/mg, less than about 0.6 EU/mg, less than about 0.5 EU/mg, less than about 0.4 EU/mg, less than about 0.3 EU/mg, less than about 0.2 EU/mg, less than about 0.1 EU/mg or less endotoxin units per mg of purified CT-1 polypeptide. The endotoxin levels or profile may be assessed at room temperature (20°C-25°C) or at body temperature (37°C).

The invention additionally provides oncofactor antibodies having improved stability as compared to existing antibodies. Stability can generally be defined as the propensity of the molecule to remain in its folded and active state. Naturally occurring molecules are usually of limited stability as their metabolism, and often their fast metabolism, is a key characteristic of their intrinsic mechanism of action in the body.

Usually, a stable protein in its folded and native structure cannot be degraded by proteases or other mechanisms. It is due to two key off pathways from the stable state by which proteins are usually eliminated in the body. These two are unfolding and aggregation. They are usually linked. Unfolding is the pathway of reverting the folded active molecule into a less folded state. Aggregation is the result of misfolding such that the molecule irreversibly turns into a non-active state. Both unfolding and aggregation significantly increase the protein's susceptibility to proteolytic or other digestion. The present invention provides a modified folding and unfolding pathway of an oncofactor antibody such that the resulting entity is more stable than an oncofactor antibody that is not produced by the methods of the invention.

In particular embodiments, the invention provides an antibody composition having increased stability against insoluble protein aggregate formation. "Protein aggregate" or "protein aggregation" is used herein to refer to protein that is no longer in solution. While protein aggregate can refer to agglomeration or oligomerization of two or more individual protein molecules, it is not limited to such a definition. Protein aggregates, as used in the art, can be soluble or insoluble; however, for the purposes of particular embodiments of the invention, protein aggregates are usually considered to be insoluble, unless otherwise specifically noted. Insoluble aggregates whose formation should be prevented in the process according to the

invention are essentially understood as protein aggregates having a size of at least 1 μm but can also be in the range above 10 μm. The particles can be determined by suitable particle counting methods using commercial particle counting instruments such as, for example, the particle counting instrument AccuSizer 700 from PSS (Particle Sizing Systems, USA) or a Pacific Scientific HIAC Royco liquid particle counting system, model 9703, equipped with a LD400 laser counter. According to the USP (US-Pharmacopoeia) a maximum of 6000 particles in the range above 10 μm and a maximum of 600 particles in the range above 25 μm are allowed per injected dose of a pharmaceutical preparation. This can be achieved according to the invention to provide for therapeutic compositions of oncofactor antibodies.

In a particular embodiments, the a composition comprising one or more oncofactor antibodies has increased stability against aggregate formation induced by one or more freeze/thaw cycles, agitation stress, or one or more outside physical or chemical stresses including non-limiting examples of heat stress, chemical stress (*e.g.,* pH, low/high salt, and the like), fluid stress (*e.g.*, compression stresses, such as those caused by fluid movement through constricted openings). As used herein "agitation stress" is taken to mean any physical movement applied to the composition either passively or actively. Non-limiting examples of agitation stresses, include bumping, dropping, shaking, swirling, vortexing, decanting, injecting, withdrawing (as into a syringe from a containing or vessel), and the like. The compositions of the invention are particularly stabilized with respect to the forces of shipping and transportation.

Oncofactor antibodies having improved stability may retain 90% residual activity at a temperature that is 2-10 degrees higher than exsting antibodies. The percentage of residual (*i.e.*, folded, active) protein may be measured by routine biochemical techniques such as HPLC, SDS PAGE or by activity assays such as binding assays or eliciting a response from cells.

In particular embodiments, the present invention contemplates compositions comprising cancer-associated antibodies, wherein the antibodies are stable for at least 6 hours, at least 7 hours, at least 8 hours, at least 9 hours, at least 10 hours, at least 11 hours, at least 12 hours, at least 15 hours, at least 18 hours, at least 21

hours, at least 24 hours, at least 48 hours, or more, at about 37°C compared to existing antibodies that are not formulated according to the methods of the present invention.

The term "solubility" refers to the property of an agent provided herein to dissolve in a liquid solvent and form a homogeneous solution. Solubility is typically expressed as a concentration, either by mass of solute per unit volume of solvent (g of solute per kg of solvent, g per dL (100 mL), mg/mL, etc.), molarity, molality, mole fraction or other similar descriptions of concentration. The maximum equilibrium amount of solute that can dissolve per amount of solvent is the solubility of that solute in that solvent under the specified conditions, including temperature, pressure, pH, and the nature of the solvent. In certain embodiments, solubility is measured at physiological pH. In certain embodiments, solubility is measured in water or a physiological buffer such as PBS. In certain embodiments, solubility is measured in a biological fluid (solvent) such as blood or serum. In certain embodiments, the temperature can be about room temperature (*e.g.*, about 20, 21, 22, 23, 24, 25°C) or about body temperature (37°C). In certain embodiments, an agent such as a CT-1 polypeptide of the invention has a solubility of at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, or 30 mg/mL at room temperature (20°C -25°C) or at 37°C.

Protein characteristics including purity, solubility and degree of aggregation can be assessed using protein-based analytical assays and methods. Protein purity can be assessed a number of ways. For instance, purity can be assessed based on primary structure, higher order structure, size, charge, hydrophobicity, and glycosylation. Examples of methods for assessing primary structure include N- and C-terminal sequencing and peptide-mapping (see, *e.g.*, Allen et al., Biologicals. 24:255-275, 1996)). Examples of methods for assessing higher order structure include circular dichroisim (see, *e.g.*, Kelly et al., Biochim Biophys Acta. 1751:119-139, 2005), fluorescent spectroscopy (see, *e.g.*, Meagher et al., J. Biol. Chem. 273:23283-89, 1998), FT-IR, amide hydrogen-deuterium exchange kinetics, differential scanning calorimetry, NMR spectroscopy, immunoreactivity with conformationally sensitive antibodies. Higher order structure can also be assessed as a function of a variety of parameters such as pH, temperature, or added salts.

Examples of methods for assessing protein characteristics such as size include analytical ultracentrifugation and size exclusion HPLC (SEC-HPLC, or alternatively, HPLC-SEC), and exemplary methods for measuring charge include ion-exchange chromatography and isolectric focusing. Hydrophobicity can be assessed, for example, by reverse-phase HPLC and hydrophobic interaction chromatography HPLC. Glycosylation can affect pharmacokinetics (*e.g.*, clearance), conformation or stability, receptor binding, and protein function, and can be assessed, for example, by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

Certain embodiments include the use of SEC-HPLC to assess protein characteristics such as purity, size (*e.g.*, size homogeneity) or degree of aggregation, and/or to purify proteins, among other uses. SEC, also including gel-filtration chromatography (GFC) and gel-permeation chromatography (GPC), refers to a chromatographic method in which molecules in solution are separated in a porous material based on their size, or more specifically their hydrodynamic volume, diffusion coefficient, and/or surface properties. The process is generally used to separate biological molecules, and to determine molecular weights and molecular weight distributions of polymers. Typically, a biological or protein sample (such as a protein extract produced according to the protein expression methods provided herein and known in the art) is loaded into a selected size-exclusion column with a defined stationary phase (the porous material), preferably a phase that does not interact with the proteins in the sample. In certain aspects, the stationary phase is composed of inert particles packed into a dense three-dimensional matrix within a glass or steel column. The mobile phase can be pure water, an aqueous buffer, an organic solvent, or a mixture thereof. The stationary-phase particles typically have small pores and/or channels which only allow molecules below a certain size to enter. Large particles are therefore excluded from these pores and channels, and their limited interaction with the stationary phase leads them to elute as a "totally-excluded" peak at the beginning of the experiment. Smaller molecules, which can fit into the pores, are removed from the flowing mobile phase, and the time they spend immobilized in the stationary-phase pores depends, in part, on how far into the pores they penetrate. Their removal from the mobile phase flow causes them to take longer to elute from the column and results in a

separation between the particles based on differences in their size. A given size exclusion column has a range of molecular weights that can be separated. Overall, molecules larger than the upper limit will not be trapped by the stationary phase, molecules smaller than the lower limit will completely enter the solid phase and elute as a single band, and molecules within the range will elute at different rates, defined by their properties such as hydrodynamic volume. For examples of these methods in practice with pharmaceutical proteins, see Bruner *et al.*, *Journal of Pharmaceutical and Biomedical Analysis*. 15: 1929-1935, 1997.

Protein purity for clinical applications is also discussed, for example, by Anicetti *et al.* (*Trends in Biotechnology*. 7:342-349, 1989). More recent techniques for analyzing protein purity include, without limitation, the LabChip GXII, an automated platform for rapid analysis of proteins and nucleic acids, which provides high throughput analysis of titer, sizing, and purity analysis of proteins. In certain non-limiting embodiments, clinical grade proteins such as protein fragments and antibodies can be obtained by utilizing a combination of chromatographic materials in at least two orthogonal steps, among other methods (*see, e.g.*, Therapeutic Proteins: Methods and Protocols. Vol. 308, Eds., Smales and James, Humana Press Inc., 2005).

In certain embodiments, compositions comprising one or more oncofactor antibody have a purity of at least about 90%, with respect to the antibody and as measured according to routine techniques in the art. In certain embodiments, such as diagnostic compositions or certain therapeutic compositions, the antibody compositions of the invention have a purity of at least about 95%. In specific embodiments, such as therapeutic or pharmaceutical compositions, the antibody compositions of the invention have a purity of at least about 97% or 98% or 99%. In other embodiments, such as when being used as reference or research reagents, antibody of the invention can be of lesser purity, and may have a purity of at least about 70%, 75%, 80%, or 85%. Purity can be measured overall or in relation to selected components, such as other proteins, *e.g.*, purity on a protein basis.

Protein solubility assays are also included. Such assays can be utilized, for example, to determine optimal growth and purification conditions for recombinant production, to optimize the choice of buffer(s), and to optimize the choice of antibodies.

Solubility or aggregation can be evaluated according to a variety of parameters, including temperature, pH, salts, and the presence or absence of other additives. Examples of solubility screening assays include, without limitation, microplate-based methods of measuring protein solubility using turbidity or other measure as an end point, high-throughput assays for analysis of the solubility of purified recombinant proteins (see, *e.g.*, Stenvall *et al.*, Biochim Biophys Acta. 1752:6-10, 2005), assays that use structural complementation of a genetic marker protein to monitor and measure protein folding and solubility in vivo (*see, e.g.*, Wigley et al., Nature Biotechnology. 19:131-136, 2001), and electrochemical screening of recombinant protein solubility in *Escherichia coli* using scanning electrochemical microscopy (SECM) (*see, e.g.*, Nagamine et al., Biotechnology and Bioengineering. 96:1008-1013, 2006), among others.

In particular embodiments, a human therapeutic composition is provided, comprising a modified polypeptide of the invention or fragment thereof as described elsewhere herein and a pharmokinetic (PK) modulator. As used herein, the term "pharmacokinetic modulator" generally refers to an antibody modification that increases the pharmacokinetic parameters of the antibody, including, without limitation, half-life, solubility, stability, activity compared to an antibody that lacks the PK modulator. In one embodiment, the PK modulator comprises a biocompatible polymer conjugated to the antibody, including for example, polyethylene glycol (PEG).

In certain preferred embodiments, the pharmaceutical compositions of the invention comprise isolated antibodies or antigen-binding fragments thereof that specifically bind at least one cancer-associated polypeptide of the present invention, such as a cancer-associated polypeptide of any one of SEQ ID NOs: 1-24.

In certain other embodiments, the pharmaceutical compositions of the invention may comprise immunogenic polynucleotides and/or polypeptides compositions of the invention for use in prophylactic or theraputic vaccine applications. Vaccine preparation is generally described in, for example, M.F. Powell and M.J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (NY, 1995). Generally, such compositions will comprise one or more polynucleotide

and/or polypeptide compositions of the present invention in combination with one or more immunostimulants.

In one embodiment, a pharmaceutical composition comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more oncofactor antibodies or antigen binding fragments thereof. The antibodies may be directed to the same or different oncofactors.

In still other embodiments, the pharmaceutical compositions of the invention may comprise polynucleotides (*e.g.*, antisense, ribozyme, RNAi or siRNA sequences) that are effective for inhibiting the expression of one or more cancer-associated polynucleotide sequences of the invention.

It will be apparent that any of the pharmaceutical compositions described herein can contain pharmaceutically acceptable salts of the polynucleotides and polypeptides of the invention. Such salts can be prepared, for example, from pharmaceutically acceptable non-toxic bases, including organic bases (*e.g.,* salts of primary, secondary and tertiary amines and basic amino acids) and inorganic bases (*e.g.,* sodium, potassium, lithium, ammonium, calcium and magnesium salts).

In other embodiments, illustrative immunogenic compositions, *e.g.*, vaccine compositions, of the present invention comprise DNA encoding one or more of the cancer-associated polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the polynucleotide may be administered within any of a variety of delivery systems known to those of ordinary skill in the art. Indeed, numerous gene delivery techniques are well known in the art, such as those described by Rolland, *Crit. Rev. Therap. Drug Carrier Systems 15*:143-198, 1998, and references cited therein. Appropriate polynucleotide expression systems will, of course, contain the necessary regulatory DNA regulatory sequences for expression in a patient (such as a suitable promoter and terminating signal). Alternatively, bacterial delivery systems may involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope.

Therefore, in certain embodiments, polynucleotides encoding immunogenic polypeptides described herein are introduced into suitable mammalian host cells for expression using any of a number of known viral-based systems. In one

illustrative embodiment, retroviruses provide a convenient and effective platform for gene delivery systems. A selected nucleotide sequence encoding a polypeptide of the present invention can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to a subject. A number of illustrative retroviral systems have been described (*e.g.*, U.S. Pat. No. 5,219,740; Miller and Rosman (1989) *BioTechniques 7*:980-990; Miller, A. D. (1990) *Human Gene Therapy 1*:5-14; Scarpa *et al.* (1991) *Virology 180*:849-852; Burns *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:8033-8037; and Boris-Lawrie and Temin (1993) *Cur. Opin. Genet. Develop. 3*:102-109.

In addition, a number of illustrative adenovirus-based systems have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham (1986) *J. Virol. 57*:267-274; Bett *et al.* (1993) *J. Virol. 67*:5911-5921; Mittereder *et al.* (1994) *Human Gene Therapy 5*:717-729; Seth *et al.* (1994) *J. Virol. 68*:933-940; Barr *et al.* (1994) *Gene Therapy 1*:51-58; Berkner, K. L. (1988) *BioTechniques 6*:616-629; and Rich *et al.* (1993) *Human Gene Therapy 4*:461-476).

Various adeno-associated virus (AAV) vector systems have also been developed for polynucleotide delivery. AAV vectors can be readily constructed using techniques well known in the art. *See*, *e.g.*, U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 and WO 93/03769; Lebkowski *et al.* (1988) *Molec. Cell. Biol. 8*:3988-3996; Vincent *et al.* (1990) *Vaccines 90* (Cold Spring Harbor Laboratory Press); Carter, B. J. (1992) *Current Opinion in Biotechnology 3*:533-539; Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol. 158*:97-129; Kotin, R. M. (1994) *Human Gene Therapy 5*:793-801; Shelling and Smith (1994) *Gene Therapy 1*:165-169; and Zhou *et al.* (1994) *J. Exp. Med. 179*:1867-1875.

Additional viral vectors useful for delivering the polynucleotides encoding polypeptides of the present invention by gene transfer include those derived from the pox family of viruses, such as vaccinia virus and avian poxvirus. By way of example, vaccinia virus recombinants expressing the novel molecules can be constructed as follows. The DNA encoding a polypeptide is first inserted into an

appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the polypeptide of interest into the viral genome. The resulting TK.sup.(-) recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

A vaccinia-based infection/transfection system can be conveniently used to provide for inducible, transient expression or coexpression of one or more polypeptides described herein in host cells of an organism. In this particular system, cells are first infected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide or polynucleotides of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into polypeptide by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, *e.g.*, Elroy-Stein and Moss, *Proc. Natl. Acad. Sci. USA 87*:6743-6747 (1990); Fuerst *et al.*, *Proc. Natl. Acad. Sci. USA 83*:8122-8126 (1986).

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the coding sequences of interest. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an Avipox vector is particularly desirable in human and other mammalian species since members of the Avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant Avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. *See*, *e.g.*, WO 91/12882; WO 89/03429; and WO 92/03545.

Any of a number of alphavirus vectors can also be used for delivery of polynucleotide compositions of the present invention, such as those vectors described in U.S. Patent Nos. 5,843,723; 6,015,686; 6,008,035 and 6,015,694. Certain vectors based on Venezuelan Equine Encephalitis (VEE) can also be used, illustrative examples of which can be found in U.S. Patent Nos. 5,505,947 and 5,643,576.

Moreover, molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael *et al. J. Biol. Chem. 268*:6866-6869 (1993) and Wagner *et al.*, *Proc. Natl. Acad. Sci. USA 89*:6099-6103 (1992), can also be used for gene delivery under the invention.

Additional illustrative information on these and other known viral-based delivery systems can be found, for example, in Fisher-Hoch *et al.*, *Proc. Natl. Acad. Sci. USA 86*:317-321, 1989; Flexner *et al.*, *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner *et al.*, *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld *et al.*, *Science 252*:431-434, 1991; Kolls *et al.*, *Proc. Natl. Acad. Sci. USA 91*:215-219, 1994; Kass-Eisler *et al.*, *Proc. Natl. Acad. Sci. USA 90*:11498-11502, 1993; Guzman *et al.*, *Circulation 88*:2838-2848, 1993; and Guzman *et al.*, *Cir. Res. 73*:1202-1207, 1993.

In certain embodiments, a polynucleotide may be integrated into the genome of a target cell. This integration may be in the specific location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the polynucleotide may be stably maintained in the cell as a separate, episomal segment of DNA. Such polynucleotide segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. The manner in which the expression construct is delivered to a cell and where in the cell the polynucleotide remains is dependent on the type of expression construct employed.

In another embodiment of the invention, a polynucleotide is administered/delivered as "naked" DNA, for example as described in Ulmer *et al.*, *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993.

The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

In still another embodiment, a composition of the present invention can be delivered via a particle bombardment approach, many of which have been described. In one illustrative example, gas-driven particle acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI), some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This approach offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, such as polynucleotide or polypeptide particles, are accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest.

In a related embodiment, other devices and methods that may be useful for gas-driven needle-less injection of compositions of the present invention include those provided by Bioject, Inc. (Portland, OR), some examples of which are described in U.S. Patent Nos. 4,790,824; 5,064,413; 5,312,335; 5,383,851; 5,399,163; 5,520,639 and 5,993,412.

According to another embodiment, the pharmaceutical compositions described herein will comprise one or more immunostimulants in addition to the immunogenic polynucleotide, polypeptide, antibody, T-cell, TCR, and/or APC compositions of this invention. An immunostimulant refers to essentially any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. One preferred type of immunostimulant comprises an adjuvant. Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine;

acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

Certain preferred adjuvants for eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with an aluminum salt. MPL® adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato *et al.*, *Science 273*:352, 1996. Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins . Other preferred formulations include more than one saponin in the adjuvant combinations of the present invention, for example combinations of at least two of the following group comprising QS21, QS7, Quil A, β-escin, or digitonin.

Alternatively the saponin formulations may be combined with vaccine vehicles composed of chitosan or other polycationic polymers, polylactide and polylactide-co-glycolide particles, poly-N-acetyl glucosamine-based polymer matrix, particles composed of polysaccharides or chemically modified polysaccharides, liposomes and lipid-based particles, particles composed of glycerol monoesters, *etc.* The saponins may also be formulated in the presence of cholesterol to form particulate structures such as liposomes or ISCOMs. Furthermore, the saponins may be formulated together with a polyoxyethylene ether or ester, in either a non-particulate solution or suspension, or in a particulate structure such as a paucilamelar liposome or ISCOM. The saponins may also be formulated with excipients such as Carbopol^{R} to increase

viscosity, or may be formulated in a dry powder form with a powder excipient such as lactose.

In one embodiment, the adjuvant system includes the combination of a monophosphoryl lipid A and a saponin derivative, such as the combination of QS21 and 3D-MPL® adjuvant, as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. Another particularly preferred adjuvant formulation employing QS21, 3D-MPL® adjuvant and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Another enhanced adjuvant system involves the combination of a CpG-containing oligonucleotide and a saponin derivative particularly the combination of CpG and QS21 is disclosed in WO 00/09159. Preferably the formulation additionally comprises an oil in water emulsion and tocopherol.

Additional illustrative adjuvants for use in the pharmaceutical compositions of the invention include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (*e.g.,* SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox (Enhanzyn®) (Corixa, Hamilton, MT), RC-529 (Corixa, Hamilton, MT) and other aminoalkyl glucosaminide 4-phosphates (AGPs), such as those described in pending U.S. Patent Application Serial Nos. 08/853,826 and 09/074,720, the disclosures of which are incorporated herein by reference in their entireties, and polyoxyethylene ether adjuvants such as those described in WO 99/52549A1.

Other preferred adjuvants include adjuvant molecules of the general formula

(I): $HO(CH_2CH_2O)_n$-A-R,

wherein, n is 1-50, A is a bond or –C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl.

One embodiment of the present invention consists of a vaccine formulation comprising a polyoxyethylene ether of general formula (I), wherein *n* is between 1 and 50, preferably 4-24, most preferably 9; the *R* component is C_{1-50}, preferably C_4-C_{20} alkyl and most preferably C_{12} alkyl, and *A* is a bond. The concentration of the polyoxyethylene ethers should be in the range 0.1-20%, preferably

from 0.1-10%, and most preferably in the range 0.1-1%. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether, polyoxyethylene-9-steoryl ether, polyoxyethylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12^{th} edition: entry 7717). These adjuvant molecules are described in WO 99/52549.

The polyoxyethylene ether according to the general formula (I) above may, if desired, be combined with another adjuvant. For example, a preferred adjuvant combination is preferably with CpG as described in the pending UK patent application GB 9820956.2.

According to another embodiment of this invention, an immunogenic composition described herein is delivered to a host via antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically compatible with the receiver (*i.e.,* matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will typically vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, mucosal, intravenous, intracranial, intraperitoneal, subcutaneous and intramuscular administration.

Carriers for use within such pharmaceutical compositions are biocompatible, and may also be biodegradable. In certain embodiments, the formulation preferably provides a relatively constant level of active component release. In other embodiments, however, a more rapid rate of release immediately upon

administration may be desired. The formulation of such compositions is well within the level of ordinary skill in the art using known techniques. Illustrative carriers useful in this regard include microparticles of poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose, dextran and the like. Other illustrative delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (*e.g.,* a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (*see e.g.,* U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

In another illustrative embodiment, biodegradable microspheres (*e.g.,* polylactate polyglycolate) are employed as carriers for the compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344, 5,407,609 and 5,942,252. Modified hepatitis B core protein carrier systems. such as described in WO/99 40934, and references cited therein, will also be useful for many applications. Another illustrative carrier/delivery system employs a carrier comprising particulate-protein complexes, such as those described in U.S. Patent No. 5,928,647, which are capable of inducing a class I-restricted cytotoxic T lymphocyte responses in a host.

In another illustrative embodiment, calcium phosphate core particles are employed as carriers, vaccine adjuvants, or as controlled release matrices for the compositions of this invention. Exemplary calcium phosphate particles are disclosed, for example, in published patent application No. WO/0046147.

The pharmaceutical compositions of the invention will often further comprise one or more buffers (*e.g.,* neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.,* glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a

recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate.

The pharmaceutical compositions described herein may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are typically sealed in such a way to preserve the sterility and stability of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

The development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.,* oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation, is well known in the art, some of which are briefly discussed below for general purposes of illustration.

In certain applications, the pharmaceutical compositions disclosed herein may be delivered *via* oral administration to an animal. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (see, for example, Mathiowitz *et al.*, Nature 1997 Mar 27;386(6623):410-4; Hwang *et al.*, Crit Rev Ther Drug Carrier Syst 1998;15(3):243-84; U. S. Patent 5,641,515; U. S. Patent 5,580,579 and U. S. Patent 5,792,451). Tablets, troches, pills, capsules and the like may also contain any of a variety of additional components, for example, a binder, such as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to

materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

Typically, these formulations will contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In certain circumstances it will be desirable to deliver the pharmaceutical compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally. Such approaches are well known to the skilled artisan, some of which are further described, for example, in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363. In certain embodiments, solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations generally will contain a preservative to prevent the growth of microorganisms.

Illustrative pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (for example, see U. S. Patent 5,466,468).

In one embodiment, for parenteral administration in an aqueous solution, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. Moreover, for human administration, preparations will of course preferably meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

In another embodiment of the invention, the compositions disclosed herein may be formulated in a neutral or salt form. Illustrative pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

The carriers can further comprise any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The phrase

"pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering genes, nucleic acids, and peptide compositions directly to the lungs *via* nasal aerosol sprays has been described, *e.g.,* in U. S. Patent 5,756,353 and U. S. Patent 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, J Controlled Release 1998 Mar 2;52(1-2):81-7) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Likewise, illustrative transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U. S. Patent 5,780,045.

In certain embodiments, liposomes, nanocapsules, microparticles, lipid particles, vesicles, and the like, are used for the introduction of the compositions of the present invention into suitable host cells/organisms. In particular, the compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like. Alternatively, compositions of the present invention can be bound, either covalently or non-covalently, to the surface of such carrier vehicles.

The formation and use of liposome and liposome-like preparations as potential drug carriers is generally known to those of skill in the art (see for example, Lasic, Trends Biotechnol 1998 Jul;16(7):307-21; Takakura, Nippon Rinsho 1998 Mar;56(3):691-5; Chandran *et al.*, Indian J Exp Biol. 1997 Aug;35(8):801-9; Margalit, Crit Rev Ther Drug Carrier Syst. 1995;12(2-3):233-61; U.S. Patent 5,567,434; U.S. Patent 5,552,157; U.S. Patent 5,565,213; U.S. Patent 5,738,868 and U.S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally difficult to transfect by other procedures, including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.,* J Biol Chem. 1990 Sep 25;265(27):16337-42; Muller *et al.*, DNA Cell Biol. 1990 Apr;9(3):221-9). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, various drugs,

radiotherapeutic agents, enzymes, viruses, transcription factors, allosteric effectors and the like, into a variety of cultured cell lines and animals. Furthermore, he use of liposomes does not appear to be associated with autoimmune responses or unacceptable toxicity after systemic delivery.

In certain embodiments, liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs).

Alternatively, in other embodiments, the invention provides for pharmaceutically-acceptable nanocapsule formulations of the compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (see, for example, Quintanar-Guerrero *et al.*, Drug Dev Ind Pharm. 1998 Dec;24(12):1113-28). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) may be designed using polymers able to be degraded *in vivo*. Such particles can be made as described, for example, by Couvreur *et al.,* Crit Rev Ther Drug Carrier Syst. 1988;5(1):1-20; zur Muhlen *et al.*, Eur J Pharm Biopharm. 1998 Mar;45(2):149-55; Zambaux *et al.* J Controlled Release. 1998 Jan 2;50(1-3):31-40; and U. S. Patent 5,145,684.

Cancer Therapeutic Methods

Immunologic approaches to cancer therapy are based on the recognition that cancer cells can often evade the body's defenses against aberrant or foreign cells and molecules, and that these defenses might be therapeutically stimulated to regain the lost ground, *e.g.*, pgs. 623-648 in Klein, Immunology (Wiley-Interscience, New York, 1982). Numerous recent observations that various immune effectors can directly or indirectly inhibit growth of tumors has led to renewed interest in this approach to cancer therapy, *e.g.*, Jager, *et al.*, Oncology 2001;60(1):1-7; Renner, *et al.*, Ann Hematol 2000 Dec;79(12):651-9.

Four-basic cell types whose function has been associated with antitumor cell immunity and the elimination of tumor cells from the body are: i) B-lymphocytes which secrete immunoglobulins into the blood plasma for identifying and labeling the nonself invader cells; ii) monocytes which secrete the complement proteins that are responsible for lysing and processing the immunoglobulin-coated target invader cells;

iii) natural killer lymphocytes having two mechanisms for the destruction of tumor cells, antibody-dependent cellular cytotoxicity and natural killing; and iv) T-lymphocytes possessing antigen-specific receptors and having the capacity to recognize a tumor cell carrying complementary marker molecules (Schreiber, H., 1989, in Fundamental Immunology (ed). W. E. Paul, pp. 923-955).

Cancer immunotherapy generally focuses on inducing humoral immune responses, cellular immune responses, or both. Moreover, it is well established that induction of $CD4^+$ T helper cells is necessary in order to secondarily induce either antibodies or cytotoxic $CD8^+$ T cells. Polypeptide antigens that are selective or ideally specific for cancer cells offer a powerful approach for inducing immune responses against cancer, and are an important aspect of the present invention.

Therefore, in further aspects of the present invention, the pharmaceutical compositions described herein may be administered to a subject in need thereof, such as a subject afflicted with or prone to develop cancer. Within such methods, the pharmaceutical compositions described herein are administered to a patient, typically a warm-blooded animal, preferably a human.

Pharmaceutical compositions and vaccines of the invention may be administered prior to or following surgical removal of primary tumors and/or treatment such as administration of radiotherapy or conventional chemotherapeutic drugs. As discussed above, administration of the pharmaceutical compositions may be by any suitable method, including administration by intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal, intradermal, anal, vaginal, topical and oral routes.

The cancer type to be treated according to the methods of the invention can be essentially any type with which a polypeptide of the invention is associated. In certain illustrative embodiments, for example, the cancer type to be treated using a compositions of the present invention is liver cancer, pancreatic cancer, lung cancer, breast cancer, bladder cancer, kidney cancer, skin cancer and hematological cancer.

Within certain embodiments, immunotherapy may be active immunotherapy, in which treatment relies on the *in vivo* stimulation of the endogenous host immune system to react against tumors with the administration of immune

response-modifying agents (such as polypeptides and polynucleotides as provided herein).

Within certain other embodiments, immunotherapy may be passive immunotherapy, in which treatment involves the delivery of agents with established tumor-immune reactivity (such as antibodies or effector cells) that can directly or indirectly mediate antitumor effects and does not necessarily depend on an intact host immune system.

Examples of effector cells include T cells as discussed above, T lymphocytes (such as $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T-helper tumor-infiltrating lymphocytes), killer cells (such as Natural Killer cells and lymphokine-activated killer cells), B cells and antigen-presenting cells (such as dendritic cells and macrophages) expressing a polypeptide provided herein. T cell receptors and antibody receptors specific for the polypeptides recited herein may be cloned, expressed and transferred into other vectors or effector cells for adoptive immunotherapy. The polypeptides provided herein may also be used to generate antibodies or anti-idiotypic antibodies (*e.g.*, as described above and in U.S. Patent No. 4,918,164) for passive immunotherapy using standard methodologies.

Monoclonal antibodies may be labeled with any of a variety of labels for desired selective usages in detection, diagnostic assays or therapeutic applications (as described in U.S. Patent Nos. 6,090,365; 6,015,542; 5,843,398; 5,595,721; and 4,708,930, hereby incorporated by reference in their entirety as if each was incorporated individually). In each case, the binding of the labelled monoclonal antibody to the determinant site of the antigen will signal detection or delivery of a particular therapeutic agent to the antigenic determinant on the non-normal cell. A further object of this invention is to provide the specific monoclonal antibody suitably labelled for achieving such desired selective usages thereof.

Routes and frequency of administration of the therapeutic compositions described herein, as well as dosage, will vary from individual to individual, and may be readily established using standard techniques. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally.

Preferably, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of a compound that, when administered as described above, is capable of promoting an anti-tumor immune response, and is at least 10-50% above the basal (*i.e.,* untreated) level. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial or longer disease-free survival) in vaccinated patients as compared to non-vaccinated patients. In general, for pharmaceutical compositions and vaccines comprising one or more polypeptides, the amount of each polypeptide present in a dose ranges from about 25 μg to 5 mg per kg of host. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial, or longer disease-free survival) in treated patients as compared to non-treated patients. Increases in preexisting immune responses to a tumor protein generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

Cancer Detection and Diagnostic Compositions, Methods and Kits

The cancer-associated sequences and binding agents of the present invention can also be used in the context of cancer diagnostic compositions, methods and kits.

In general, a cancer may be detected in a patient based on the presence of one or more cancer-associated polypeptides and/or polynucleotides encoding such polypeptipdes in a biological sample (for example, blood, sera, sputum urine and/or

tumor biopsies) obtained from the patient. In other words, such proteins may be used as markers to indicate the presence or absence of a cancer.

In some embodiments, polynucleotide primers and probes may be used to detect the level of mRNA encoding a cancer-associated protein, which is also indicative of the presence or absence of a cancer. In general, a cancer-associated sequence may be present at a level that is at least two-fold, preferably three-fold, and more preferably five-fold or higher in tumor tissue than in normal tissue of the same type from which the tumor arose. Expression levels of a particular tumor sequence in tissue types different from that in which the tumor arose are irrelevant in certain diagnostic embodiments since the presence of tumor cells can be confirmed by observation of predetermined differential expression levels, *e.g.*, 2-fold, 5-fold, etc, in tumor tissue to expression levels in normal tissue of the same type.

Other differential expression patterns can be utilized advantageously for diagnostic purposes. For example, in one aspect of the invention, overexpression of a tumor sequence in tumor tissue and normal tissue of the same type, but not in other normal tissue types, *e.g.,* PBMCs, can be exploited diagnostically. In this case, the presence of metastatic tumor cells, for example in a sample taken from the circulation or some other tissue site different from that in which the tumor arose, can be identified and/or confirmed by detecting expression of the tumor sequence in the sample, for example using RT-PCR analysis. In many instances, it will be desired to enrich for tumor cells in the sample of interest, *e.g.*, PBMCs, using cell capture or other like techniques.

There are a variety of assay formats known to those of ordinary skill in the art for using a binding agent to detect polypeptide markers in a sample. *See*, *e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In general, the presence or absence of a cancer in a patient may be determined by (a) contacting a biological sample obtained from a patient with a binding agent; (b) detecting in the sample a level of polypeptide that binds to the binding agent; and (c) comparing the level of polypeptide with a predetermined cut-off value.

In an illustrative embodiment, the assay involves the use of binding agent immobilized on a solid support to bind to and remove the polypeptide from the

remainder of the sample. The bound polypeptide may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/polypeptide complex. Such detection reagents may comprise, for example, a binding agent that specifically binds to the polypeptide or an antibody or other agent that specifically binds to the binding agent, such as an anti-immunoglobulin, protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the binding agent is indicative of the reactivity of the sample with the immobilized binding agent. Suitable polypeptides for use within such assays include full length cancer-associated proteins and polypeptide portions thereof to which the binding agent binds, as described above.

The solid support may be any material known to those of ordinary skill in the art to which the tumor protein may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the agent and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about

10 μg, and preferably about 100 ng to about 1 μg, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner (*see, e.g.*, Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that polypeptides within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the polypeptide) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with cancer at least about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of

time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited above.

The detection reagent is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of a cancer, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one embodiment, the cut-off value for the detection of a cancer is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without the cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value is considered positive for the cancer. In an alternate embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett *et al.*, *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, p. 106-7. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible

cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for a cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the binding agent is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the immobilized binding agent as the sample passes through the membrane. A second, labeled binding agent then binds to the binding agent-polypeptide complex as a solution containing the second binding agent flows through the membrane. The detection of bound second binding agent may then be performed as described above. In the strip test format, one end of the membrane to which binding agent is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second binding agent and to the area of immobilized binding agent. Concentration of second binding agent at the area of immobilized antibody indicates the presence of a cancer. Typically, the concentration of second binding agent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of binding agent immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferred binding agents for use in such assays are antibodies and antigen-binding fragments thereof. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1μg, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use with the tumor proteins or binding agents of the present invention. The above descriptions are intended to be exemplary only. For example, it will be apparent to those of ordinary skill in the art that the above protocols may be readily modified to use tumor polypeptides to detect antibodies that bind to such polypeptides in a biological sample. The detection of such tumor protein specific antibodies may correlate with the presence of a cancer.

A cancer may also, or alternatively, be detected based on the presence of T cells that specifically react with a cancer-associated protein of the invention in a biological sample. Within certain methods, a biological sample comprising $CD4^+$ and/or $CD8^+$ T cells isolated from a patient is incubated with a tumor polypeptide, a polynucleotide encoding such a polypeptide and/or an APC that expresses at least an immunogenic portion of such a polypeptide, and the presence or absence of specific activation of the T cells is detected. Suitable biological samples include, but are not limited to, isolated T cells. For example, T cells may be isolated from a patient by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes). T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with polypeptide (*e.g.*, 5-25 μg/ml). It may be desirable to incubate another aliquot of a T cell sample in the absence of tumor polypeptide to serve as a control. For $CD4^+$ T cells, activation is preferably detected by evaluating proliferation of the T cells. For $CD8^+$ T cells, activation is preferably detected by evaluating cytolytic activity. A level of proliferation that is at least two fold greater and/or a level of cytolytic activity that is at least 20% greater than in disease-free patients indicates the presence of a cancer in the patient.

As noted above, a cancer may also, or alternatively, be detected based on the level of mRNA encoding a cancer-associated protein of the present invention in a biological sample. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify a portion of a tumor cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for (*i.e.,* hybridizes to) a polynucleotide encoding the tumor protein.

The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis.

Similarly, oligonucleotide probes that specifically hybridize to a polynucleotide encoding a cancer-associated protein may be used in a hybridization assay to detect the presence of polynucleotide encoding the cancer-associated protein in a biological sample.

To permit hybridization under assay conditions, oligonucleotide primers and probes should comprise an oligonucleotide sequence that has at least about 60%, preferably at least about 75% and more preferably at least about 90%, identity to a portion of a polynucleotide encoding a cancer-associated protein of the invention that is at least 10 nucleotides, and preferably at least 20 nucleotides, in length. Preferably, oligonucleotide primers and/or probes hybridize to a polynucleotide encoding a polypeptide described herein under moderately stringent conditions, as defined above. Oligonucleotide primers and/or probes which may be usefully employed in the diagnostic methods described herein preferably are at least 10-40 nucleotides in length. In one embodiment, the oligonucleotide primers comprise at least 10 contiguous nucleotides, more preferably at least 15 contiguous nucleotides, of a DNA molecule having a sequence as disclosed herein. Techniques for both PCR based assays and hybridization assays are well known in the art (*see*, for example, Mullis *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, *51*:263, 1987; Erlich ed., *PCR Technology*, Stockton Press, NY, 1989).

One assay employs RT-PCR, in which PCR is applied in conjunction with reverse transcription. Typically, RNA is extracted from a biological sample, such as biopsy tissue, and is reverse transcribed to produce cDNA molecules. PCR amplification using at least one specific primer generates a cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification may be performed on biological samples taken from a test patient and from an individual who is not afflicted with a cancer. The amplification reaction may be performed on several dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the test patient sample as compared to the same dilutions of the non-cancerous sample is typically considered positive.

In another aspect of the present invention, cell capture technologies may be used in conjunction, with, for example, real-time PCR to provide a more sensitive tool for detection of metastatic cells expressing tumor antigens. Detection of cancer cells in biological samples, *e.g.*, bone marrow samples, peripheral blood, and small needle aspiration samples is desirable for diagnosis and prognosis in cancer patients.

Immunomagnetic beads coated with specific monoclonal antibodies to surface cell markers, or tetrameric antibody complexes, may be used to first enrich or positively select cancer cells in a sample. Various commercially available kits may be used, including Dynabeads® Epithelial Enrich (Dynal Biotech, Oslo, Norway), StemSep™ (StemCell Technologies, Inc., Vancouver, BC), and RosetteSep (StemCell Technologies). A skilled artisan will recognize that other methodologies and kits may also be used to enrich or positively select desired cell populations. Dynabeads® Epithelial Enrich contains magnetic beads coated with mAbs specific for two glycoprotein membrane antigens expressed on normal and neoplastic epithelial tissues. The coated beads may be added to a sample and the sample then applied to a magnet, thereby capturing the cells bound to the beads. The unwanted cells are washed away and the magnetically isolated cells eluted from the beads and used in further analyses.

RosetteSep can be used to enrich cells directly from a blood sample and consists of a cocktail of tetrameric antibodies that targets a variety of unwanted cells and crosslinks them to glycophorin A on red blood cells (RBC) present in the sample, forming rosettes. When centrifuged over Ficoll, targeted cells pellet along with the free RBC. The combination of antibodies in the depletion cocktail determines which cells will be removed and consequently which cells will be recovered. Antibodies that are available include, but are not limited to: CD2, CD3, CD4, CD5, CD8, CD10, CD11b, CD14, CD15, CD16, CD19, CD20, CD24, CD25, CD29, CD33, CD34, CD36, CD38, CD41, CD45, CD45RA, CD45RO, CD56, CD66B, CD66e, HLA-DR, IgE, and TCR$\alpha\beta$.

Additionally, it is contemplated in the present invention that mAbs specific for tumor antigens can be generated and used in a similar manner. For example, mAbs that bind to tumor-specific cell surface antigens may be conjugated to magnetic beads, or formulated in a tetrameric antibody complex, and used to enrich or

positively select metastatic tumor cells from a sample. Once a sample is enriched or positively selected, cells may be lysed and RNA isolated. RNA may then be subjected to RT-PCR analysis using tumor-specific primers in a real-time PCR assay as described herein. One skilled in the art will recognize that enriched or selected populations of cells may be analyzed by other methods (*e.g., in situ* hybridization or flow cytometry).

In another embodiment, the compositions described herein may be used as markers for the progression of cancer. In this embodiment, assays as described above for the diagnosis of a cancer may be performed over time, and the change in the level of reactive polypeptide(s) or polynucleotide(s) evaluated. For example, the assays may be performed every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In general, a cancer is progressing in those patients in whom the level of polypeptide or polynucleotide detected increases over time. In contrast, the cancer is not progressing when the level of reactive polypeptide or polynucleotide either remains constant or decreases with time.

Certain *in vivo* diagnostic assays may be performed directly on a tumor. One such assay involves contacting tumor cells with a binding agent. The bound binding agent may then be detected directly or indirectly via a reporter group. Such binding agents may also be used in histological applications. Alternatively, polynucleotide probes may be used within such applications.

As noted above, to improve sensitivity, multiple tumor protein markers may be assayed within a given sample. It will be apparent that binding agents specific for different proteins provided herein may be combined within a single assay. Further, multiple primers or probes may be used concurrently. The selection of tumor protein markers may be based on routine experiments to determine combinations that results in optimal sensitivity. In addition, or alternatively, assays for tumor proteins provided herein may be combined with assays for other known tumor antigens.

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof that specifically binds to a tumor

protein. Such antibodies or fragments may be provided attached to a support material, as described above. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively, contain a detection reagent as described above that contains a reporter group suitable for direct or indirect detection of antibody binding.

Alternatively, a kit may be designed to detect the level of mRNA encoding a tumor protein in a biological sample. Such kits generally comprise at least one oligonucleotide probe or primer, as described above, that hybridizes to a polynucleotide encoding a tumor protein. Such an oligonucleotide may be used, for example, within a PCR or hybridization assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or container to facilitate the detection of a polynucleotide encoding a tumor protein.

Screening Assays for Drug Candidates

Screening assays for drug candidates may be designed to identify compounds that bind to or complex with the cancer-associated polypeptides of the invention, or otherwise interfere with the interaction of the polypeptides with other cellular proteins. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates. Small molecules contemplated include synthetic organic or inorganic compounds, including peptides, preferably soluble peptides, (poly)peptide-immunoglobulin fusions, and, in particular, antibodies including, without limitation, poly- and monoclonal antibodies and antibody fragments, single-chain antibodies, anti-idiotypic antibodies, and chimeric or humanized versions of such antibodies or fragments, as well as human antibodies and antibody fragments. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art. All assays are common in that they call for contacting the drug candidate with a polypeptide of the invention under conditions and for a time sufficient to allow these two components to interact.

In binding assays, the interaction is binding and the complex formed can be isolated or detected in the reaction mixture. In a particular embodiment, the

polypeptide of the invention or the drug candidate is immobilized on a solid phase, *e.g.*, on a microtiter plate, by covalent or non-covalent attachments. Non-covalent attachment generally is accomplished by coating the solid surface with a solution of the polypeptide and drying. Alternatively, an immobilized antibody, *e.g.*, a monoclonal antibody, specific for the polypeptide to be immobilized can be used to anchor it to a solid surface. The assay is performed by adding the non-immobilized component, which may be labeled by a detectable label, to the immobilized component, *e.g.*, the coated surface containing the anchored component. When the reaction is complete, the non-reacted components are removed, *e.g.*, by washing, and complexes anchored on the solid surface are detected. When the originally non-immobilized component carries a detectable label, the detection of label immobilized on the surface indicates that complexing occurred. Where the originally non-immobilized component does not carry a label, complexing can be detected, for example, by using a labelled antibody specifically binding the immobilized complex.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

GENERATION AND CHARACTERIZATION OF ANTIBODIES AGAINST ONCOFACTORS

As noted, the present invention relates, in certain aspects, to pharmaceutical compositions comprising isolated antibodies and antigen-binding fragments thereof that specifically bind an oncofactor sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2), IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO: 19), SEMA4B (SEQ ID NO: 20), SEMA4D

(SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24). Such compositions can be made and used according to the general disclosure herein and further in view of the illustrative examples set out below.

a. Characterization of expression in cell lines

Baseline expression of oncofactor sequences is characterized by RT-PCR in representative cell lines, including cell lines: IM-9 (B cell lymphoma), 4T1 (breast cancer carcinoma), C1498 (acute myeloid leukemia), and TRAMP-C2 (prostate carcinoma).

Real-time PCR (*see* Gibson *et al.*, *Genome Research 6*:995-1001, 1996; Heid *et al.*, *Genome Research 6*:986-994, 1996) is a technique that evaluates the level of PCR product accumulation during amplification. This technique permits quantitative evaluation of mRNA levels in multiple samples. Briefly, in one illustrative approach, mRNA is extracted from tumor and normal tissue and cDNA is prepared using standard techniques. Real-time PCR is performed, for example, using a Perkin Elmer/Applied Biosystems (Foster City, CA) 7700 Prism instrument. Matching primers and fluorescent probes are designed for genes of interest using, for example, the primer express program provided by Perkin Elmer/Applied Biosystems (Foster City, CA). Optimal concentrations of primers and probes are initially determined by those of ordinary skill in the art, and control (*e.g.,* β-actin) primers and probes are obtained commercially from, for example, Perkin Elmer/Applied Biosystems (Foster City, CA). To quantitate the amount of specific RNA in a sample, a standard curve is typically generated using a plasmid containing the gene of interest. Standard curves are generated using the Ct values determined in the real-time PCR, which are related to the initial cDNA concentration used in the assay. Standard dilutions ranging from 10-10^6 copies of the gene of interest are generally sufficient. In addition, a standard curve is generated for the control sequence. This permits standardization of initial RNA content of a tissue sample to the amount of control for comparison purposes.

An alternative real-time PCR procedure can be carried out as follows: The first-strand cDNA to be used in the quantitative real-time PCR is synthesized from 20μg of total RNA that is first treated with DNase I (*e.g.*, Amplification Grade, Gibco BRL Life Technology, Gaitherburg, MD), using Superscript Reverse Transcriptase

(RT) (*e.g.*, Gibco BRL Life Technology, Gaitherburg, MD). Real-time PCR is performed, for example, with a GeneAmp™ 5700 sequence detection system (PE Biosystems, Foster City, CA). The 5700 system uses SYBR™ green, a fluorescent dye that only intercalates into double stranded DNA, and a set of gene-specific forward and reverse primers. The increase in fluorescence is monitored during the whole amplification process. The optimal concentration of primers is determined using a checkerboard approach and a pool of cDNAs from tumors is used in this process. The PCR reaction is performed in 25µl volumes that include 2.5µl of SYBR green buffer, 2µl of cDNA template and 2.5µl each of the forward and reverse primers for the gene of interest. The cDNAs used for RT reactions are diluted approximately 1:10 for each gene of interest and 1:100 for the β-actin control. In order to quantitate the amount of specific cDNA (and hence initial mRNA) in the sample, a standard curve is generated for each run using the plasmid DNA containing the gene of interest. Standard curves are generated using the Ct values determined in the real-time PCR which are related to the initial cDNA concentration used in the assay. Standard dilution ranging from 20-2x10^6 copies of the gene of interest are used for this purpose. In addition, a standard curve is generated for β-actin ranging from 200fg-2000fg. This enables standardization of the initial RNA content of a tissue sample to the amount of β-actin for comparison purposes. The mean copy number for each group of tissues tested is normalized to a constant amount of β-actin, allowing the evaluation of the over-expression levels seen with each of the genes.

b. Generation of cell lines that vary by expression

For cell lines that are baseline low/negative, oncofactor polypeptides are ectopically overexpressed by cloning the coding sequence into a suitable vector, such as the retrovirally expression vector pMXs-IP, and using that vector to transduce the cell lines. Positively transduced cell lines are selected for with puromycin, as the vector encodes a puromycin resistance cassette, and overexpression is confirmed by RT-PCR.

For cell lines that are baseline positive, cancer associated polypeptide expression is knocked down with RNAi. shRNA constructs that target the oncofactor sequence are cloned into the HuSH vector (Origene, Rockville, MD) and delivered to

target cells via shRNA transduction. Knockdown of the cancer associated sequences is confirmed by RT-PCR.

c. Characterization of effects on cell proliferation and morphology

Cell lines which are positive or negative for expression of the oncofactor sequence(s) are plated at equal density and counted every day for seven days. Simultaneously, cells are observed for morphological changes that vary with oncofactor protein expression.

d. Characterization of antibody binding

Antibodies targeting the oncofactor protein sequences are characterized for binding by western blotting. Lysates of cells that are positive or negative for expression are run on SDS-PAGE gel and blotted with the antibody. In cases where the oncofactor protein is a secreted protein, supernatants from cells expressing the protein are collected, proteins are precipitated with trichloroacetic acid, run on SDS-PAGE, and western blotted with the antibody. In cases where the protein is bound to the cell surface, antibody binding may also be confirmed by flow cytometry. Cells which are positive for target expression are stained with the antibody, and then counterstained with a relevant sencondary antibody (which is specific to the constant region of the primary antibody, and which is conjugated to a fluorescent protein. Antibody binding is then visualized in the fluorescence channel of the cytometer.

e. Antibody dependent cell cytotoxicity (ADCC) assays

Target cell lines which are positive or negative for oncofactor sequence expression will be loaded with Calcein-AM reagent (Beckton Dickinson, Sparks, MD) which is cleaved by intracellular esterases and retained within the cell cytosol. Cells are incubated with antibody specific to the oncofactor protein or an isotype control. Cells are then mixed in varying ratios with human PBMCs from a healthy adult volunteer which have been isolated by Ficoll gradient. Following 4 hours incubation, supernatants are collected, and time resolved fluorescence is measured as a readout for cell lysis, and thus ADCC.

f. Complement dependent cytotoxicity (CDC) assay

Target cell lines which are positive or negative for oncofactor protein expression will be loaded with Calcein AM reagent which is cleaved by intracellular

esterases and retained within the cell cytosol. Cells are incubated with antibody specific to the oncofactor protein or an isotype control. Cells are then mixed with varying dilutions of fresh human serum from a healthy adult volunteer. Following 4 hours of incubation, supernatants are collected and time resolved fluorescence is measured as a readout for cell lysis, and thus CDC.

g. Mixed lymphocyte reactions

Raji B lymphoma cells which are positive or negative for oncofactor protein expression will be treated with mitomicin-C to inhibit proliferation and activation. Raji B cells are then incubated with peripheral blood mononuclear cells (PBMCs) isolated by Ficoll density gradient from whole blood of adult healthy volunteers. The mixed cell cultures are incubated together for various lengths of time and in varying cell ratios.

Cytokine analysis: Supernatants from the mixed cultures are collected and cytokine release by the bulk mixed lymphocyte cultures is assessed by commercially available ELISA reagents. Cytokines examined include IL-1β, IL-2, IL-4, IL-6, IL-10, IFNγ, TNF, and TGFβ. Additionally, cytokines released by specific cell populations will be assessed by intracellular cytokine staining in conjunction with linage marker staining and assessed by flow cytometry. Specific cell populations will be defined as Th1 T cells: CD3+, CD4+, Tbet+, CD8-, CD19-, CD11b-; Th2 T cells: CD3+, CD4+, GATA3+, CD19-, CD11b-, Treg T cells: CD3+, CD4+, Foxp3+, CD19-, CD11b-; Th17 T cells: CD3+, CD4+, CD8-, RORγT+, CD19-, CD11b-; CD8 T cells: CD3+, CD4-, CD8+, CD19-, CD11b-, B cells: CD3-, CD19+, CD11b-, dendritic cells: CD3-, CD19-, CD11b+, CD11c+; macrophages: CD3-, CD19-, CD11b+, CD11c-; NK cells: asialo-GM1+, CD3-, CD19-, and CD11b-.

Activation marker expression: Additionally, at the same time points, cells are collected from the mixed cultures, stained for lineage markers and evaluated for upregulation of immune activation markers by flow cytometry. Activation markers include (for T cells): CD25, CD44, CD69, and CD154; (for antigen presenting cells including B cells): CD40, CD80, CD86, MHCII; and (for NK cells): CD69 and CD161.

Proliferation of responding PBMCs: PBMC populations are assessed by staining the PBMCs with CFSE prior to incubation with the Raji B cells. At various

time points after coculture, proliferation of the PBMCs is assessed by dilution of CFSE in specific populations, as delineated by surface markers. Stained PBMCs that are not cocultured with Raji B cells are used as a negative control.

Cytotoxic activity assay: Raji B cells positive or negative for oncofactor protein expression are incubated with PBMCs for six days. Subsequently, PBMC/ Raji B coculture are added to fresh Raji B cells that have been loaded with Calcein-AM reagent. Following 4 hours incubation, supernatants are collected and time resolved fluorescence is measured as a readout for lysis of the Raji B cells.

This is similar to the ADCC assays, but for these assays, the oncofactor protein-expressing Raji B cells are permitted to activate the PBMCs for several days in the absence of antibody, where in the ADCC assay, fresh PBMCs are added to oncofactor protein-expressing cells (Raji B, 4T1, C1498, or TRAMP-C2) together with antibody and cell lysis is measured after only hours.

Mixed lymphocyte reactions with antibody: Assays of the mixed lymphocyte reactions are repeated in the presence of antibody which specifically binds to the oncofactor protein. The ability of the antibody to reverse the oncofactor protein-mediated phenotype is assessed.

h. Characterization of expression in primary tumors

Antibodies that bind to an oncofactor protein by western blot (as described above) are used to characterize oncofactor protein expression on primary human tumor. Core samples from a spectrum of tumors (including but not limited to liver, pancreas, breast, lung, bladder, prostate) and matched normal tissue are stained with the antibody. The degree of antibody staining is assessed, preferably by a licensed pathologist.

EXAMPLE 2

ACTIVATION OF PBMCS WITH ONCOFACTORS INCREASES SPECIFIC LYSIS OF TUMOR CELLS

Tumor cell lysis methods

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque gradient separation from whole blood obtained by venous puncture from a

healthy donor. Raji B lymphoma cells were treated with mitomycin C to cross link DNA and prevent replication. A mixed tumor lymphocyte culture (MTLC) was performed; PBMCs were cocultured with Raji cells for 6 days at a ratio of 10:1 with recombinant oncofactors at various concentrations (see Table 2). PBMCs were activated during the 6 day coculture period to specifically recognize the Raji cells and the PBMC CD8+ T lymphocyte population was stimulated to specifically lyse the Raji cells.

On day 6, fresh Raji cells were labeled with the fluorescent calcein dye, Calcein AM, and incubated with the preactivated PBMCs for 4.5 hours. CD8+ T cell lysis of the Raji cells was detected by Calcein AM release into the supernatant, which was quantified using a fluorometer. Specific lysis was calculated as a ratio of Calcein AM release from the mixed tumor lymphocyte cultures versus Calcein AM release from Raji cells treated with Triton X-100 to achieve total lysis, after a correction for spontaneous Calcein AM release.

Specific Lysis=(MTLC-PBMC alone)/(Triton-PBMC alone)

To control for spontaneous release of Calcein AM, Raji cells were loaded with Calcein AM but were not incubated with PBMCs for the 4.5 hour lysis phase. To control for nonspecific lysis, PBMCs were cultured for an initial 6 days both without Raji cells, and, in parallel, with phytohaemagglutinin (PHA), which induces nonspecific activation. These PBMCs were added to calcein loaded Raji cells for the 4.5 hour lysis phase.

Table 2

Oncofactor	Recombinant oncofactor concentration
IL1f5	0.1 μg/ml, 1 μg/ml, 3 μg/ml
IL1RAP2	0.2 μg/ml, 2 μg/ml, 5 μg/ml
CCL14	0.5 μg/ml, 3 μg/ml, 10 μg/ml
IL1R2	0.5 μg/ml, 1 μg/ml, 3 μg/ml

Results of IL1f5 Tumor Cell Lysis Experiments

Figure 1 shows that MTLC comprising PBMCs cultured with Raji B lymphoma cells and recombinant IL1f5 polypeptide exhibit a dose dependent reduction

of Raji B lymphoma cell lysis compared to MTLC cultured without IL1f5. These results indicate that IL1f5 is capable of inhibiting immune responses. To control for nonspecific lysis, PBMCs were cultured for the initial 6 days with IL1f5 in the absence of Raji B lymphoma cells, and then added to calcein loaded Raji cells for the 4.5 hour lysis period.

Results of IL1RAP2 Tumor Cell Lysis Experiments

Figure 2 shows that MTLC comprising PBMCs cultured with Raji B lymphoma cells and recombinant IL1RAP2 polypeptide exhibit a dose dependent reduction of Raji B lymphoma cell lysis compared to MTLC cultured without IL1RAP2. These results indicate that IL1RAP2 is capable of inhibiting immune responses.

Results of CCL14 Tumor Cell Lysis Experiments

Figure 3 shows that MTLC comprising PBMCs cultured with Raji B lymphoma cells and recombinant CCL14 polypeptide exhibit a dose dependent reduction of lysis of Raji B lymphoma cells compared to MTLC cultured without CCL14. These results indicate that CCL14 is capable of inhibiting immune responses.

Results of IL1R2 Tumor Cell Lysis Experiments

Figure 18 shows that MTLC comprising PBMCs cultured with Raji B lymphoma cells and recombinant IL1R2 polypeptide exhibit a dose dependent reduction of lysis of Raji B lymphoma cells compared to MTLC cultured without IL1R2. These results indicate that IL1R2 is capable of inhibiting immune responses.

EXAMPLE 3

ONCOFACTOR IMMUNOHISTOCHEMISTRY

Immunohistochemistry Methods

Expression of individual oncofactors was evaluated in tumor and matched normal control tissue by immunohistochemistry staining. Antibodies specific to individual oncofactors was tested at various titrations to identify dilutions or concentrations that would result in minimal background and maximal signal detection. Antibody specificity was verified by staining a negative cell line, *e.g.*, HEK293T cells,

which do not express an individual oncofactor, as well as a positive cell line transiently transfected with an oncofactor, *e.g.*, HEK293T cells transfected with IL1f5 or GPR183. Oncofactor expression in the transfected HEK293T cells was verified by qPCR and western blot. Final antibody staining dilutions or concentrations are indicated in Table 3.

The primary antibodies used are indicated in Table 3. The principal detection system consisted of a Vector anti-rabbit secondary (BA-1000) and a Vector ABC-AP kit (AK-5000) with a Vector Red substrate kit (SK-5100), which produced a fuchsia-colored deposit. The negative control consisted of performing the entire immunohistochemical procedure on adjacent sections in the absence of primary antibody. Tissues were also stained with positive control antibodies (CD31 and vimentin) to ensure that tissue antigens were preserved and accessible for immunohistochemical analysis. The slides were interpreted by a pathologist and each antibody was evaluated for the presence of specific signal and level of background. Staining intensity was recorded on a 0-4 scale (0=negative, 1=blush, 2=faint, 3=moderate, 4=strong). Stained slides were imaged with a DVC 1310C digital camera coupled to a Nikon microscope. Images were stored as TIFF files with Adobe Photoshop.

Biological samples stained consisted of 40 tumor and 10 normal control tissues from each of five cancers: breast, lung, colon, pancreas, and prostate. Samples were mounted as a tissue microarray consisting of 1mm core samples from tumor biopsy along with 10 normal control tissues from the same organ.

Table 3

Oncofactor	Primary Antibody	Final Antibody concentration/dilution
IL1f5	Sigma Aldrich #HPA034542	Final dilution of 1:50
GPR183	Lifespan #LS-A46	Final Concentration of 10 μg/mL
IL1RAP	Genetex #GTX-104513	Final Concentration of 20 μg/mL
CCL14	Santa Cruz #28388	Final Concentration of 2 μg/mL

SEMA4D	Sigma Aldrich #HPA015662	Final dilution of 1:100
IL1R2	Sigma Aldrich #HPA027598	Final Dilution of 1:50

Results of IL1f5 IHC

Increased IL1f5 antibody staining was observed in tumor samples from breast, colon, lung, and prostate cancer compared to controls. Representative images are shown in Figures 4-7. IL1f5 staining was most intense in breast cancers, but was also intense in colon, lung, and prostate cancers. Furthermore, comparison of average pathology scores indicated that breast tumor samples with a higher clinical grade (indicative of more divergence from normal cell morphology) correlate with IL1f5 overexpression. The average score from Grade 3 cancers was 3.45, whereas Grade 2 cancers scored 2.31 and noncancerous tissue scored 1.12.

Results of GPR183 IHC

Increased GPR183 antibody staining was observed in tumor samples from breast, colon, and lung cancer compared to controls. Representative images are shown in Figures 8-10. GPR183 staining was most intense in breast cancers but was also intense in colon cancers and lung cancers. Furthermore, comparison of average pathology scores indicated that breast tumor samples with a higher clinical grade (indicative of more divergence from normal cell morphology) correlate with GPR183 overexpression. The average score from Grade 3 cancers was 2.32, whereas Grade 2 cancers scored 1.65 and noncancerous tissue scored 1.17.

Results of IL1RAP IHC

Increased IL1RAP antibody staining was observed in tumor samples from breast and lung cancer compared to controls. Representative images are shown in Figures 11-12. IL1f5 staining was most intense in breast cancers, but was also intense in lung cancers. The average IHC scores for malignant breast cells was 3.44, compared to 1.22 for noncancerous cells. The average IHC scores for lung tumors was 2.89, compared to 1.88 for noncancerous cells.

Results of CCL14 IHC

Increased CCL14 antibody staining was observed in tumor samples from breast, prostate, and lung cancer compared to controls. Representative images are shown in Figures 13-15. CCL14 staining was most intense in breast cancers, but was also intense in prostate, and lung cancers. The average IHC scores for malignant breast cells was 2.61, compared to 1.25 for noncancerous cells. The average IHC scores for malignant prostate cells was 1.51, compared to 0.19 for noncancerous cells. The average IHC scores for lung tumors was 1.86, compared to 0.60 for noncancerous cells.

Results of SEMA4D IHC

Increased SEMA4D antibody staining was observed in tumor samples from breast cancer compared to controls. Representative images are shown in Figure 16. SEMA4D staining was intense in breast cancers. The average IHC scores for malignant breast cells was 2.92, compared to 2.29 for noncancerous cells.

Results of IL1R2 IHC

Increased IL1R2 antibody staining was observed in tumor samples from breast cancer compared to controls. Representative images are shown in Figure 17. IL1R2 staining was intense in breast cancers. The average IHC scores for malignant breast cells was 2.25, compared to 1.5 for noncancerous cells.

EXAMPLE 4

T CELL PROLIFERATION IN THE PRESENCE OF ONCOFACTORS

Methods

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained by venous puncture from a healthy donor by Ficoll Hypaque gradient separation and labeled with carboxyfluorescein diacetate (CFSE). Raji B lymphoma cells were treated with mitomycin C to cross link DNA and prevent replication. A mixed tumor lymphocyte culture (MTLC) was then set up by coculturing PBMCs and Rajis for 6 days at a ratio of 10:1 with varying concentrations of recombinant IL1f5. During this time, T lymphocytes from the PBMC population are activated and clonally divide.

CFSE is a membrane impermeant dye. The amount of CFSE per cell will be reduced by half with each successive cell division, and the number of cell divisions can be tracked by monitoring the intensity of CFSE staining by flow cytometry. T cells were identified by positive staining with anti-CD3-PE (E Bioscience anti-CD3-PE Catalog# 17-0036-42). The number of cell division was analyzed by comparing the intensity of CFSE staining to resting T cells. T cells were defined as fully divided when the level of CFSE staining was less than the limit of detection. Data was collected on a Accuri C6 flow cytometer and analyzed with FCS Express. To control for nonspecific activation by IL1f5, PBMCs were cultured with IL1f5 in the absence of Raji cells.

Results of IL1f5 IHC

MTLC comprising PBMCs cultured with Raji B lymphoma cells and recombinant IL1f5 polypeptide resulted in a dose dependent reduction of T cell proliferation compared to MTLC cultured without IL1f5 and PBMC controls. These results (Figure 19) indicate that IL1f5 is capable of inhibiting immune responses by decreasing T cell proliferation.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

What is Claimed:

1. An isolated antibody or antigen-binding fragment that specifically binds to a sequence set forth in any one of SEQ ID NOs: 1-24, for use in the treatment of cancer.

2. An isolated polypeptide comprising a sequence set forth in any one of SEQ ID NOs: 1-24, or a fragment or variant thereof having at least 90% identity thereto, for use in the treatment of cancer.

3. An isolated polynucleotide encoding an amino acid sequence set forth in any one of SEQ ID NOs: 1-24, or encoding a fragment or variant of a sequence set forth in SEQ ID NOs: 1-24 having at least 90% identity thereto, for use in the treatment of cancer.

4. An oligonucleotide that is complementary to a polynucleotide encoding an amino acid sequence set forth in any one of SEQ ID NOs: 1-24, or encoding a fragment or variant of a sequence set forth in SEQ ID NOs: 1-24 having at least 90% identity thereto, for use in the treatment of cancer.

5. An endotoxin-free pharmaceutical composition comprising a pharmaceutically acceptable carrier and an isolated antibody or antigen-binding fragment thereof that specifically binds a sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2), IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO: 19), SEMA4B (SEQ ID NO: 20), SEMA4D

(SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24).

6. A pharmaceutical composition formulated for intravenous injection for use in a patient having or at risk for having cancer, said composition comprising a pharmaceutically acceptable carrier and an isolated antibody or antigen-binding fragment thereof that specifically binds a sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2), IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO: 19), SEMA4B (SEQ ID NO: 20), SEMA4D (SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24).

7. The composition of claim 5 or claim 6, wherein the composition comprises one or more antibodies or antigen-binding fragments thereof.

8. The composition of any one of claims 5 to 7, wherein the composition comprises one or more antibodies or antigen binding fragments thereof, wherein each of the one or more antibodies or antigen binding fragments thereof specifically bind a sequence selected from the group consisting of IL1f5 (SEQ ID NO: 1), CCBP2 (SEQ ID NO: 2), IL1R2 (SEQ ID NO: 3), IL1RAPL1 (SEQ ID NO: 4), IL18BP (SEQ ID NO: 5), CLEC2B (SEQ ID NO: 6), C4BPA (SEQ ID NO: 7), C4BPB (SEQ ID NO: 8), SERPINI1 (SEQ ID NO: 9), IL1RAP isoform 1 (SEQ ID NO: 10), IL1RAP isoform 2 (SEQ ID NO: 11), GPR1 (SEQ ID NO: 12), GPR4 (SEQ ID NO: 13), GPR15 (SEQ ID NO: 14), GPR32 (SEQ ID NO: 15), GPR34 (SEQ ID NO: 16), GPR183 (SEQ ID NO: 17), SERPINA4 (SEQ ID NO: 18), SERPINB5 (SEQ ID NO:

19), SEMA4B (SEQ ID NO: 20), SEMA4D (SEQ ID NO: 21), CCL14 (SEQ ID NO: 22), NKTR (SEQ ID NO: 23) and SFTPD (SEQ ID NO: 24).

9. The composition of claim 5, wherein the composition is 95% endtoxin free.

10. The composition of claim 5, wherein the composition is 98% endotoxin free.

11. The composition of claim 5 or claim 6, wherein the isolated antibody or antigen-binding fragment is a monoclonal antibody or antigen-binding fragment.

12. The composition of claim 5 or claim 6, wherein the isolated antibody or antigen-binding fragment is a humanized antibody or antigen-binding fragment.

13. The composition of claim 5 or claim 6, wherein the antibody or antigen binding fragment is conjugated to a toxin.

14. The composition of claim 13, wherein the toxin is selected from the group consisting of a ricin toxin, abrin toxin, diptheria toxin, cholera toxin, gelonin toxin, Pseudomonas exotoxin, Shigella toxin, and pokeweed antiviral protein.

15. The composition of claim 5 or claim 6, wherein the antibody or antigen binding fragment is a monoclonal antibody or antigen binding fragment conjugated to a radionuclide.

16. The composition of claim 15 wherein the radionuclide is selected from the group consisting of ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{211}At and ^{212}Bi.

17. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and one or more isolated polypeptide of any one of SEQ ID NOs: 1-24, or a fragment or variant thereof having at least 90% identity thereto, or an isolated polynucleotide encoding any one of the foregoing polypeptides.

18. The pharmaceutical compositions of claim 17, wherein the composition further comprises an immunostimulant.

19. A method for the treatment of cancer in a subject in need thereof comprising administering to the subject a pharmaceutical composition of any one of claims 1-14.

20. The method of claim 19 wherein the cancer is selected from liver cancer, pancreatic cancer, lung cancer, breast cancer, bladder cancer, kidney cancer, skin cancer and hematological cancer.

21. A method for detecting the presence of a cancer in a patient, comprising the steps of:

(a) obtaining a biological sample from the patient;

(b) contacting the biological sample with an antibody or antigen-binding fragment that specifically binds to a polypeptide of any one of SEQ ID NOs: 1-24;

(c) detecting in the sample an amount of polypeptide that binds to the antibody or antigen-binding fragment; and

(d) comparing the amount of polypeptide to a predetermined cut-off value and therefrom determining the presence of a cancer in the patient.

22. The method of claim 21 wherein the cancer is selected from liver cancer, pancreatic cancer, lung cancer, breast cancer, bladder cancer, kidney cancer, skin cancer and hematological cancer.

23. A method for detecting the presence of a cancer in a patient, comprising the steps of:

(a) obtaining a biological sample from the patient;

(b) contacting the biological sample with an oligonucleotide that hybridizes to a polynucleotide, wherein the polynucleotide encodes a polypeptide of any one of SEQ ID NOs: 1-24, or encodes a fragment or variant thereof having at least 90% identity thereto;

(c) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide; and

(d) comparing the amount of polynucleotide that hybridizes to the oligonucleotide to a predetermined cut-off value, and therefrom determining the presence of the cancer in the patient.

24. The method of claim 23, wherein the cancer is selected from liver cancer, pancreatic cancer, lung cancer, breast cancer, bladder cancer, kidney cancer, skin cancer and hematological cancer.

25. A diagnostic kit comprising at least one isolated antibody or antigen-binding fragment thereof that specifically binds to a sequence of any one of SEQ ID NOs: 1-24 and a detection reagent, wherein the detection reagent comprises a reporter group.

26. A diagnostic kit comprising at least one oligonucleotide that hybridizes to a polynucleotide, wherein the polynucleotide encodes a polypeptide of any one of SEQ ID NOs: 1-24, or encodes a fragment or variant thereof having at least 90% identity thereto.

27. A method for treating a cancer in a patient, comprising the steps of:

(a) detecting an amount of polypeptide of any one of SEQ ID NOs: 1-24 in a biological sample of a patient;

(b) comparing the amount of the polypeptide to a predetermined cut-off value and therefrom determining the presence of a cancer in the patient; and

(c) administering a composition according to any one of claims 1 to 14 to a subject determined to have cancer in step (b).

28. The method of claim 27, wherein the cancer is selected from liver cancer, pancreatic cancer, lung cancer, breast cancer, bladder cancer, kidney cancer, skin cancer and hematological cancer.

29. A method for treating a cancer in a patient, comprising the steps of:

(a) obtaining a biological sample from the patient;

(b) contacting the biological sample with an oligonucleotide that hybridizes to a polynucleotide, wherein the polynucleotide encodes a polypeptide of any one of SEQ ID NOs: 1-24, or encodes a fragment or variant thereof having at least 90% identity thereto;

(c) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide;

(d) comparing the amount of polynucleotide that hybridizes to the oligonucleotide to a predetermined cut-off value, and therefrom determining the presence of the cancer in the patient; and

(e) administering a composition according to any one of claims 1 to 14 to a subject determined to have cancer in step (d).

30. The method of claim 29, wherein the cancer is selected from liver cancer, pancreatic cancer, lung cancer, breast cancer, bladder cancer, kidney cancer, skin cancer and hematological cancer.

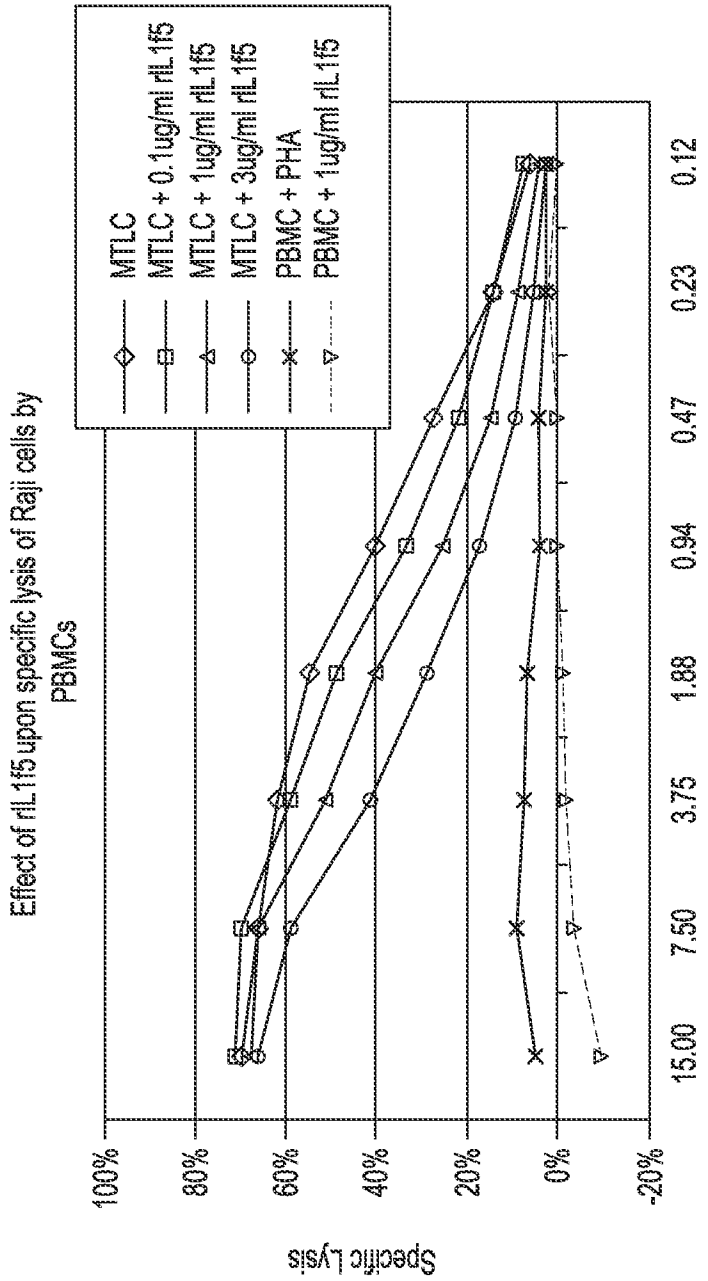
Effect of rIL15 upon specific lysis of Raji cells by PBMCs
MTLC
MTLC + 0.1ug/ml rIL15
MTLC + 1ug/ml rIL15
MTLC + 3ug/ml rIL15
PBMC + PHA
PBMC + 1ug/ml rIL15
Specific Lysis
100%
80%
60%
40%
20%
0%
-20%
15.00
7.50
3.75
1.88
0.94
0.47
0.23
0.12
PBMC: Raji Ratio

FIG. 1

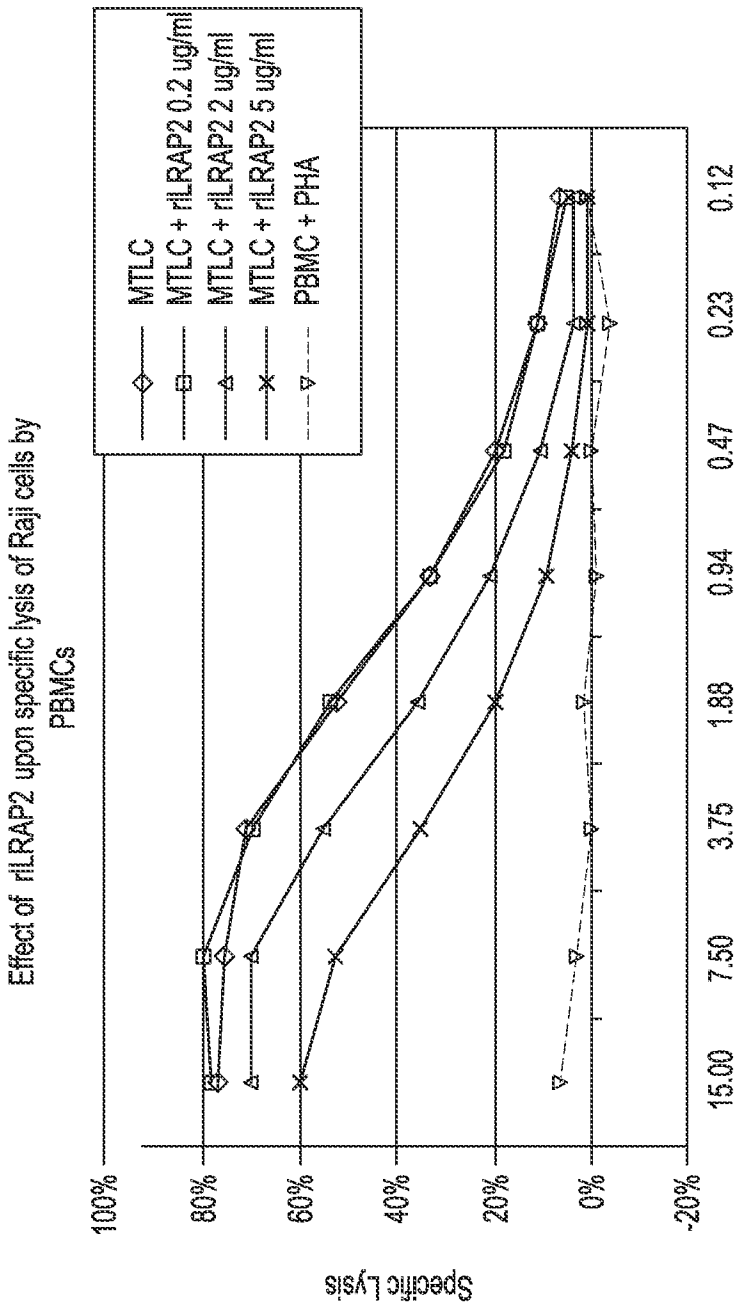
Effect of rILRAP2 upon specific lysis of Raji cells by PBMCs
MTLC
MTLC + rILRAP2 0.2 ug/ml
MTLC + rILRAP2 2 ug/ml
MTLC + rILRAP2 5 ug/ml
PBMC + PHA
Specific Lysis
100%
80%
60%
40%
20%
0%
-20%
15.00
7.50
3.75
1.88
0.94
0.47
0.23
0.12
PBMC: Raji Ratio

FIG. 2

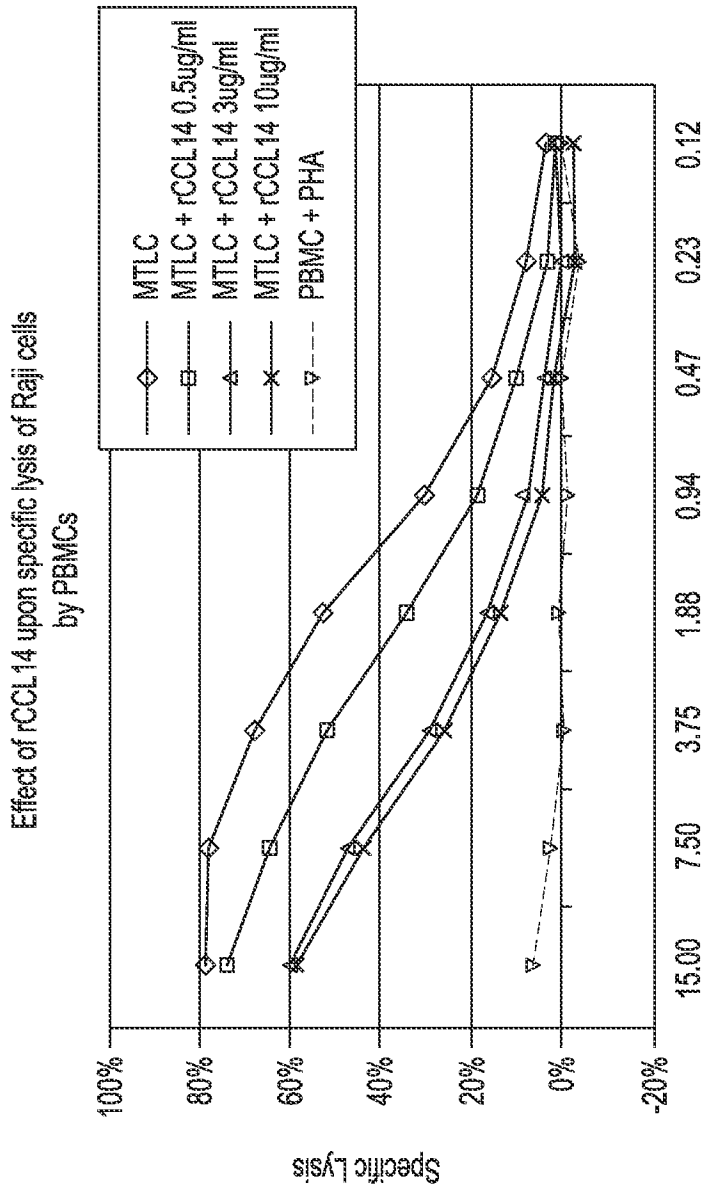
Effect of rCCL14 upon specific lysis of Raji cells by PBMCs
MTLC
MTLC + rCCL14 0.5ug/ml
MTLC + rCCL14 3ug/ml
MTLC + rCCL14 10ug/ml
PBMC + PHA
Specific Lysis
100%
80%
60%
40%
20%
0%
-20%
15.00
7.50
3.75
1.88
0.94
0.47
0.23
0.12
PBMC: Raji Ratio

FIG. 3

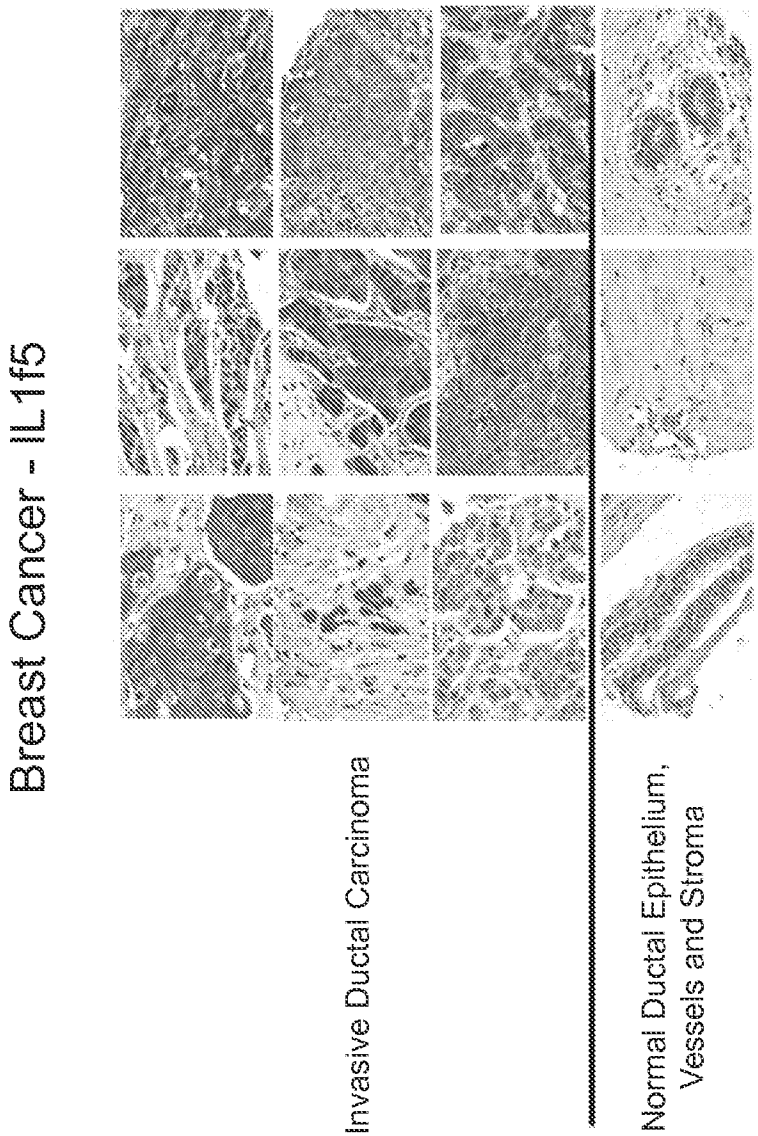
Breast Cancer - IL1f5
Invasive Ductal Carcinoma
Normal Ductal Epithelium, Vessels and Stroma

FIG. 4

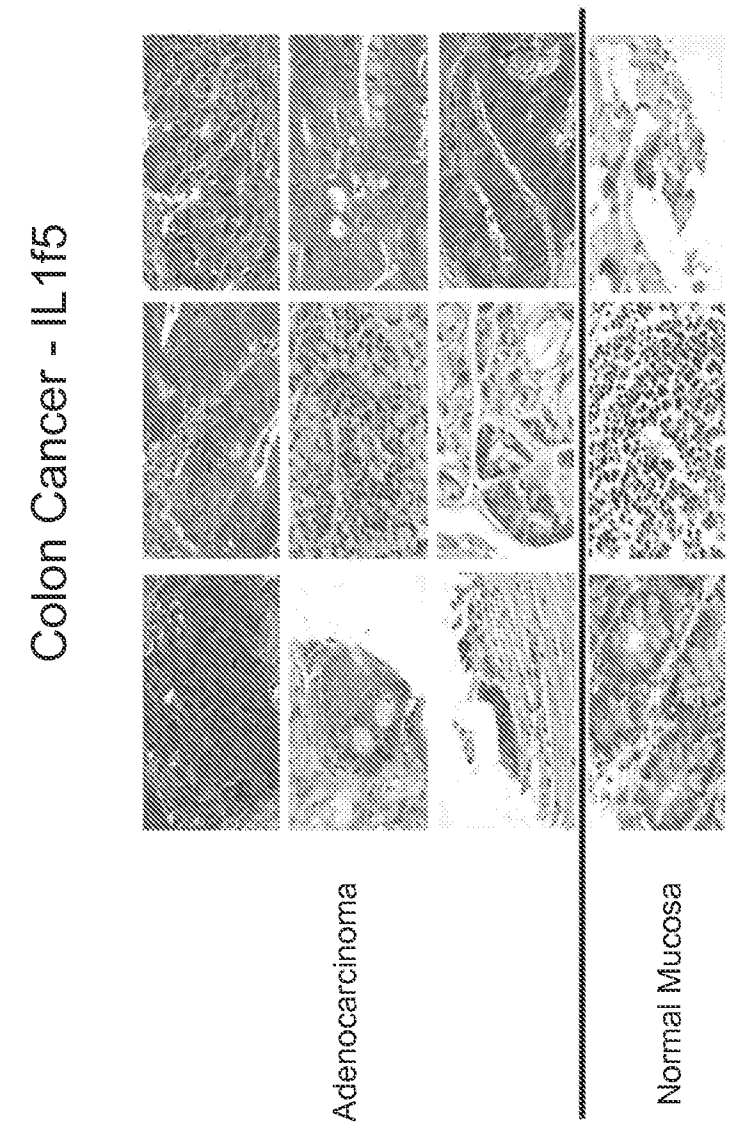

FIG. 5

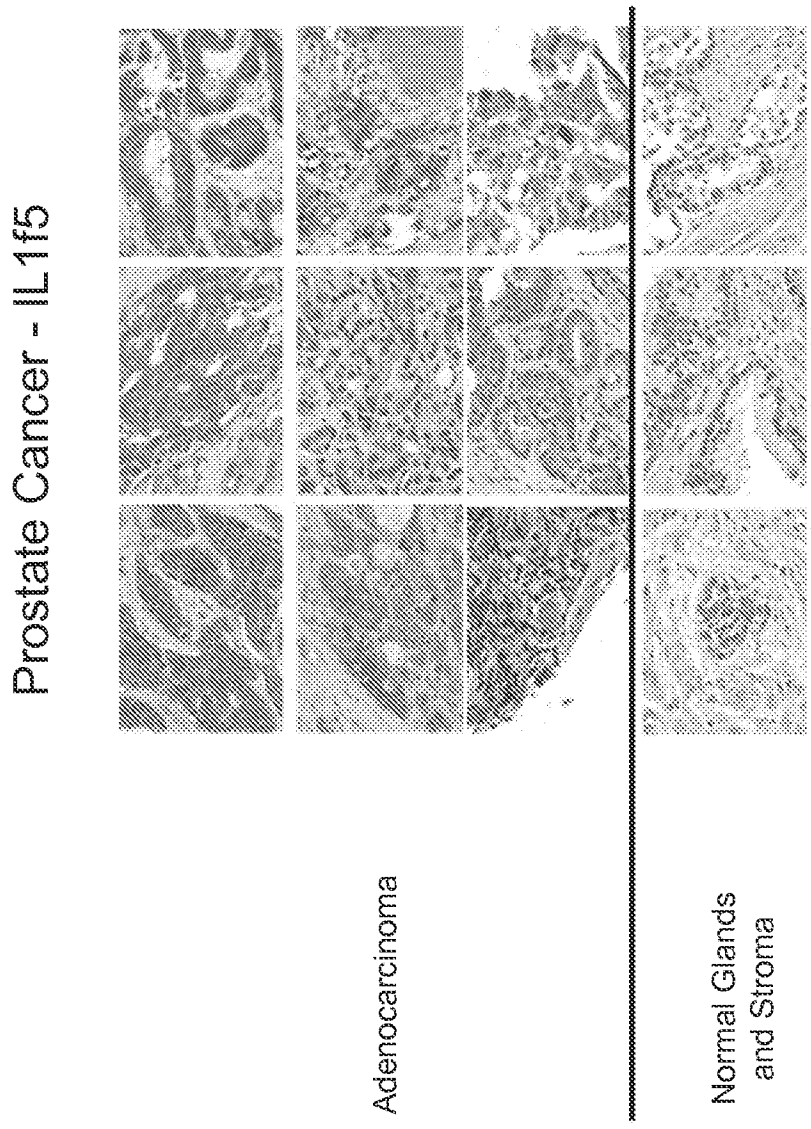
Prostate Cancer - IL1f5
Adenocarcinoma
Normal Glands and Stroma

FIG. 6

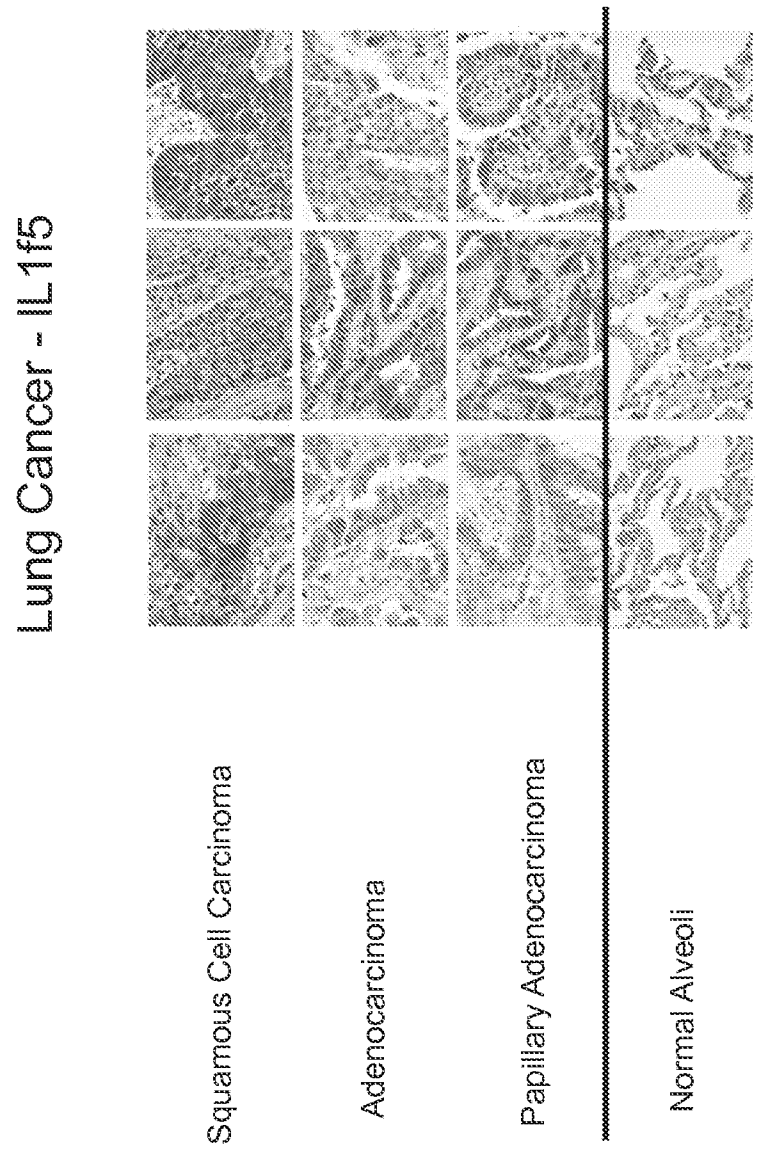
Lung Cancer - IL1f5
Squamous Cell Carcinoma
Adenocarcinoma
Papillary Adenocarcinoma
Normal Alveoli

FIG. 7

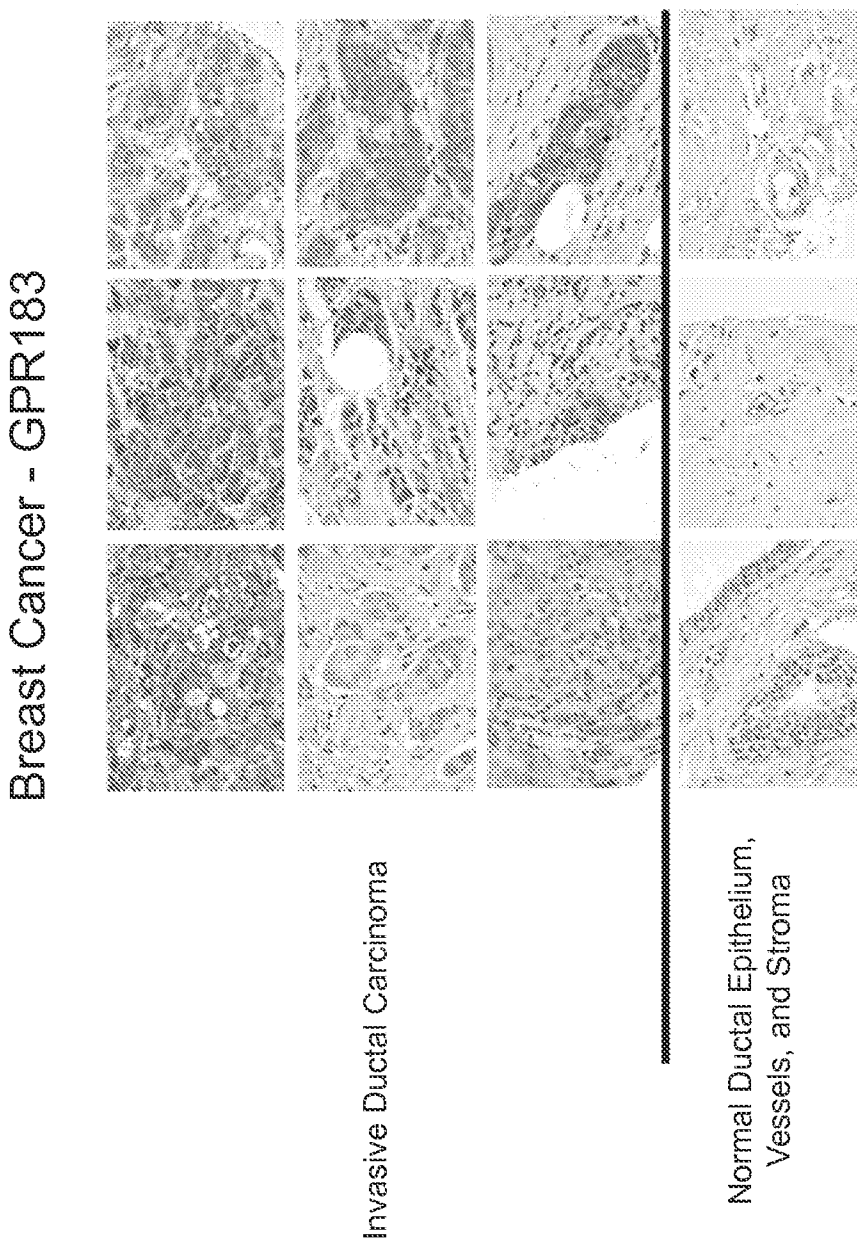
Breast Cancer - GPR183
Invasive Ductal Carcinoma
Normal Ductal Epithelium, Vessels, and Stroma

FIG. 8

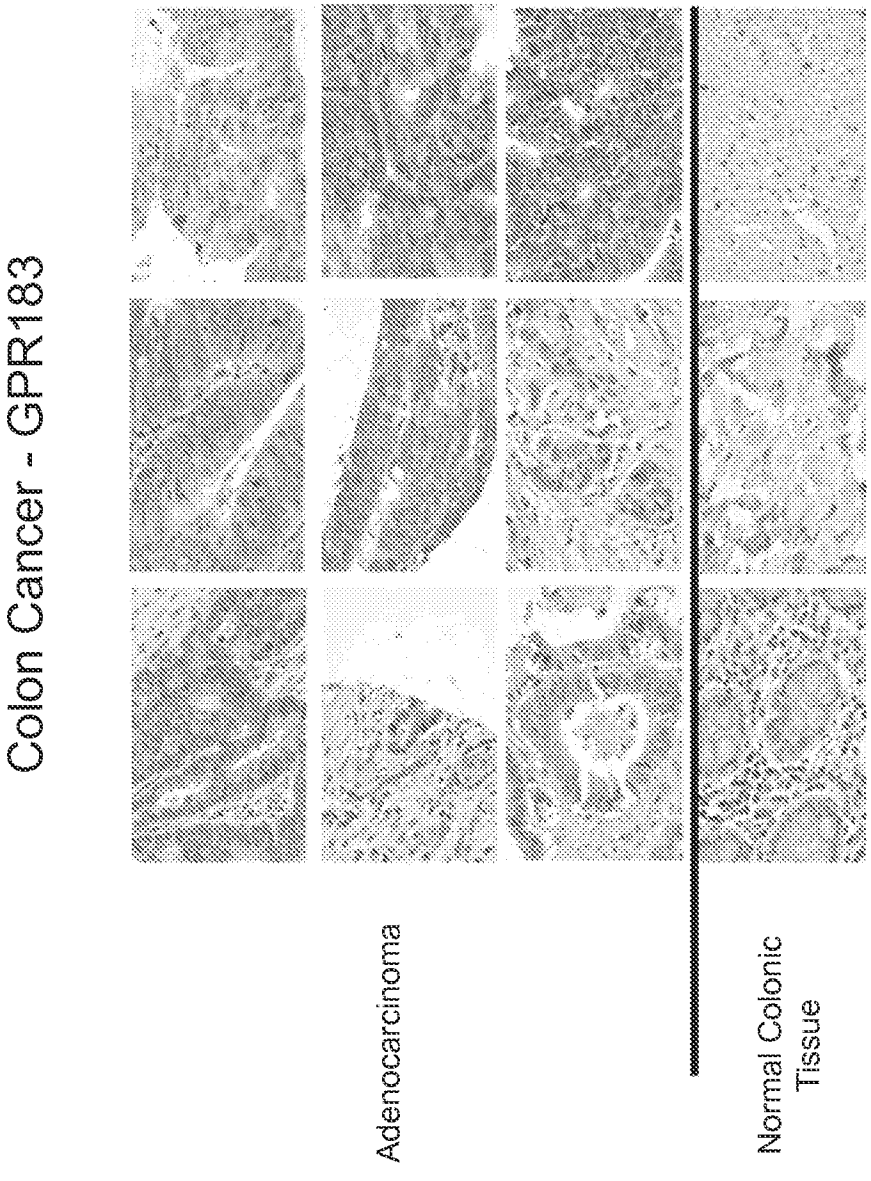
Colon Cancer - GPR183
Adenocarcinoma
Normal Colonic Tissue

FIG. 9

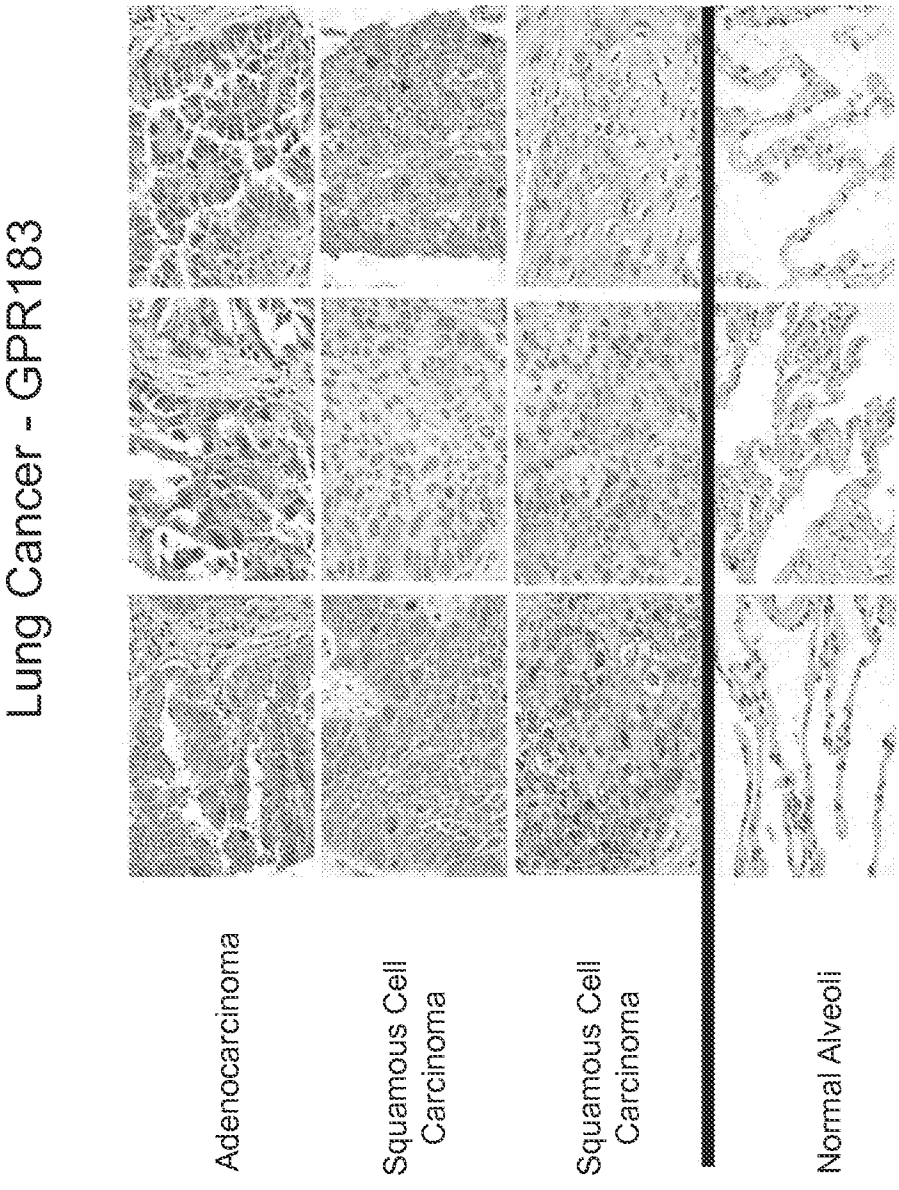
Lung Cancer - GPR183
Adenocarcinoma
Squamous Cell Carcinoma
Squamous Cell Carcinoma
Normal Alveoli

FIG. 10

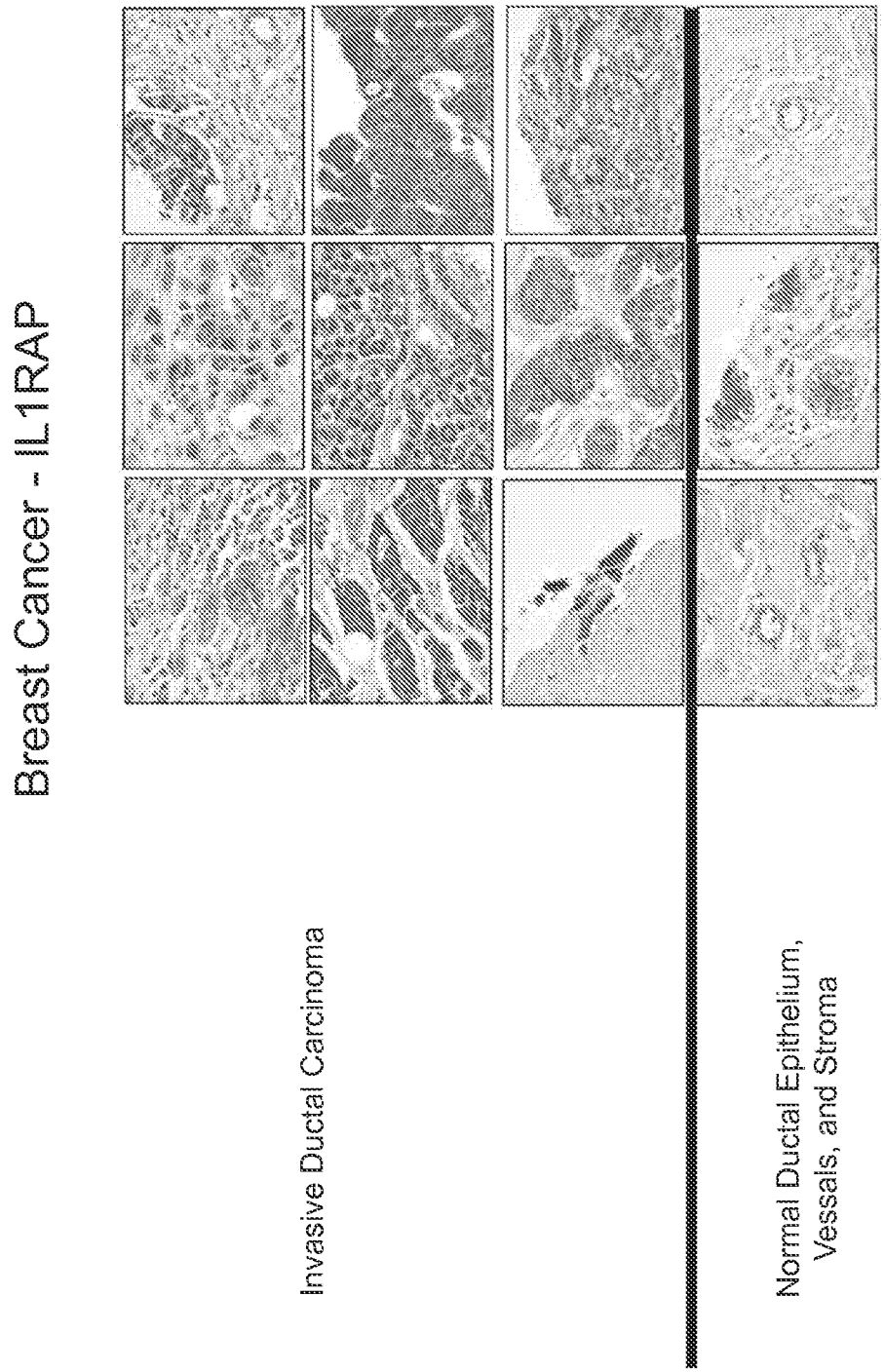
Breast Cancer - IL1RAP
Invasive Ductal Carcinoma
Normal Ductal Epithelium, Vessals, and Stroma

FIG. 11

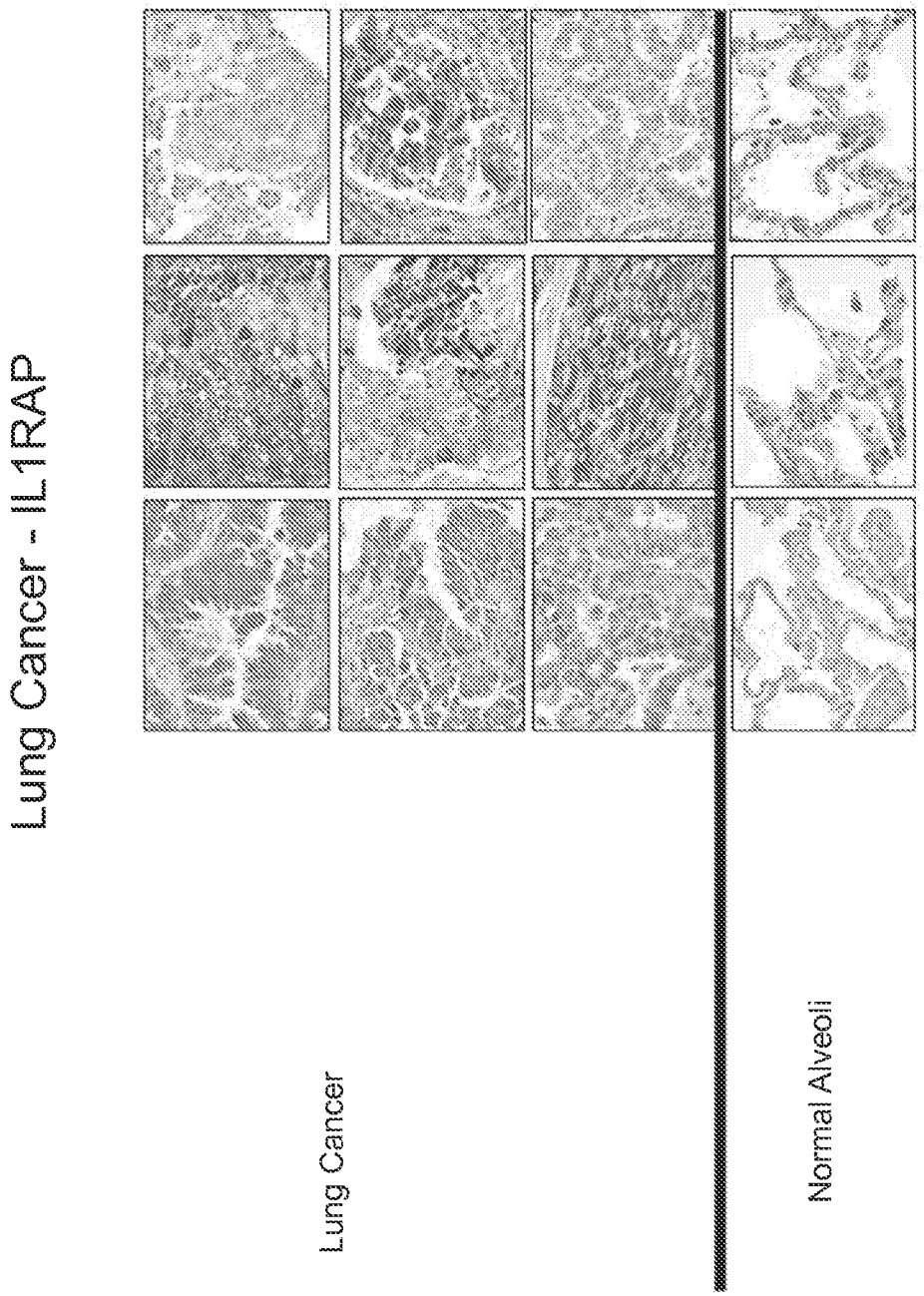

FIG. 12

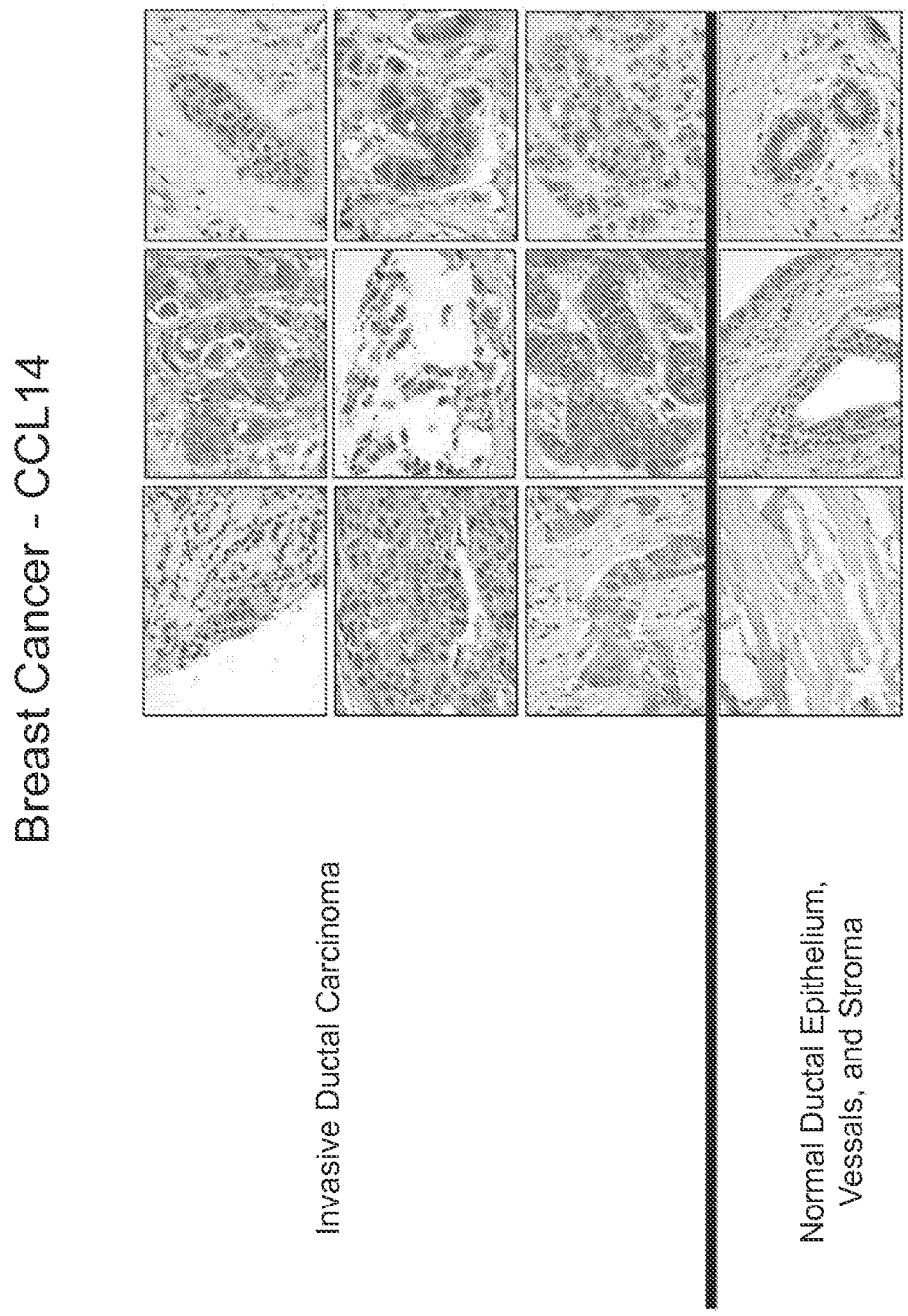
Breast Cancer - CCL14
Invasive Ductal Carcinoma
Normal Ductal Epithelium, Vessals, and Stroma

FIG. 13

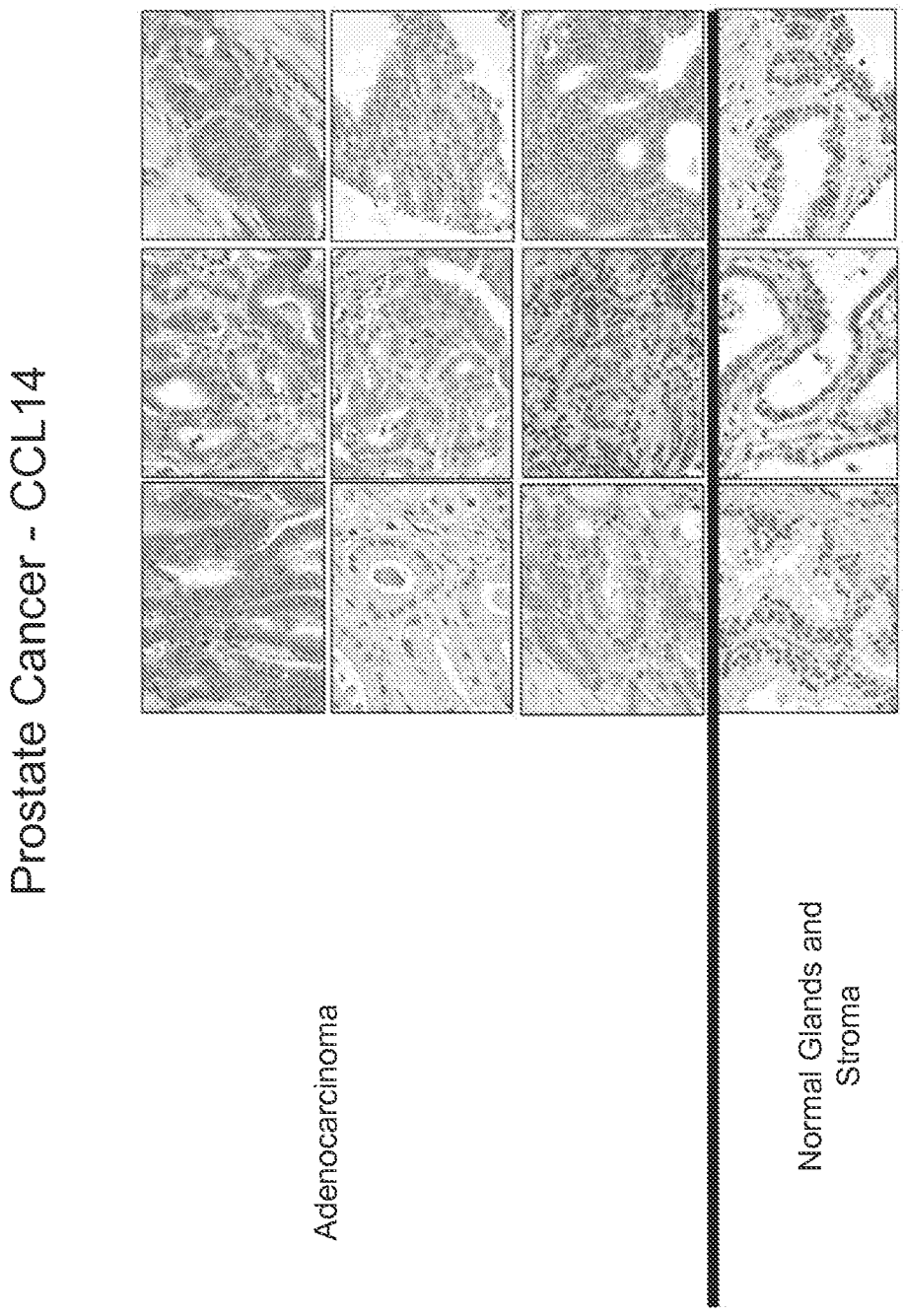
Prostate Cancer - CCL14
Adenocarcinoma
Normal Glands and Stroma

FIG. 14

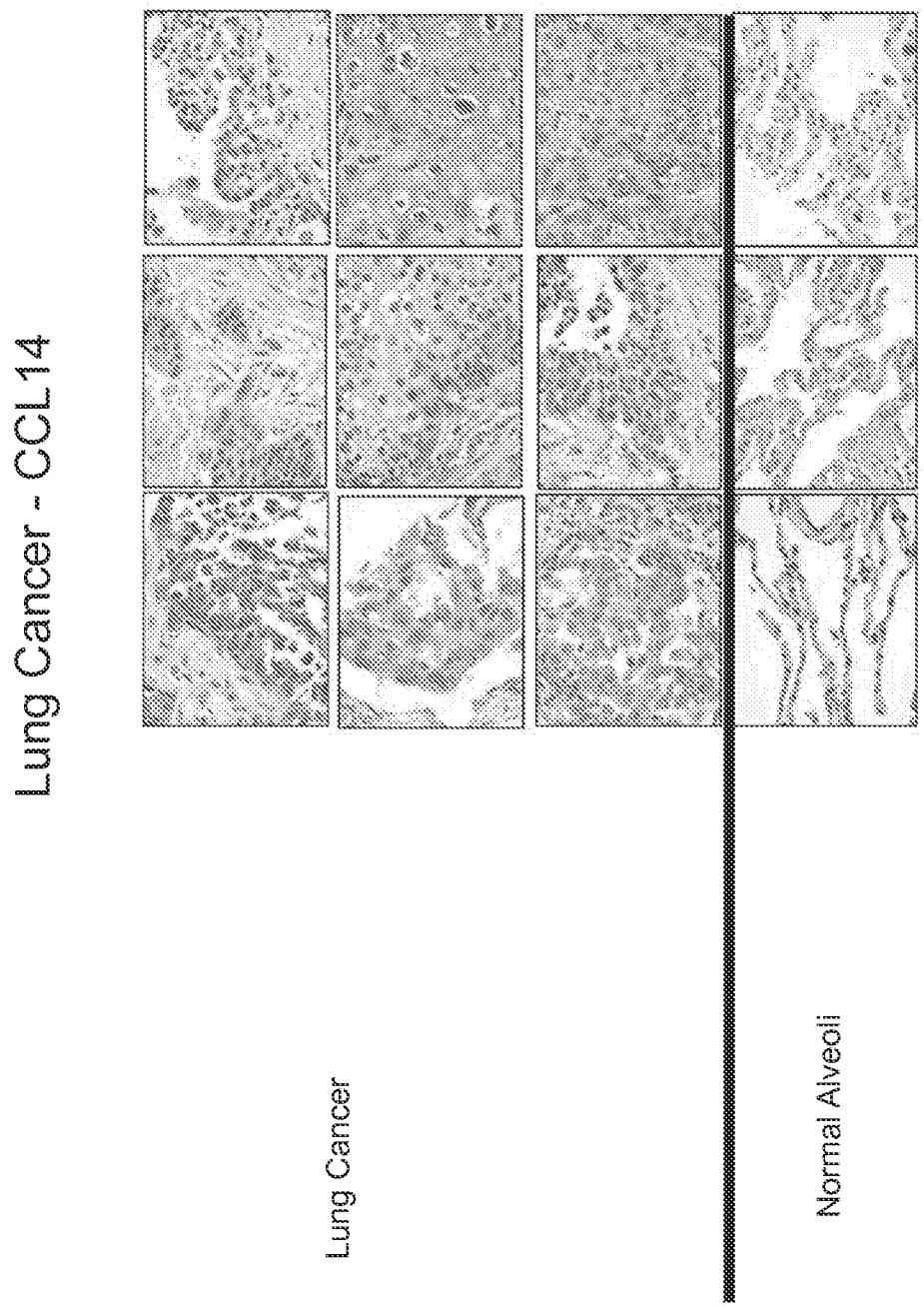

FIG. 15

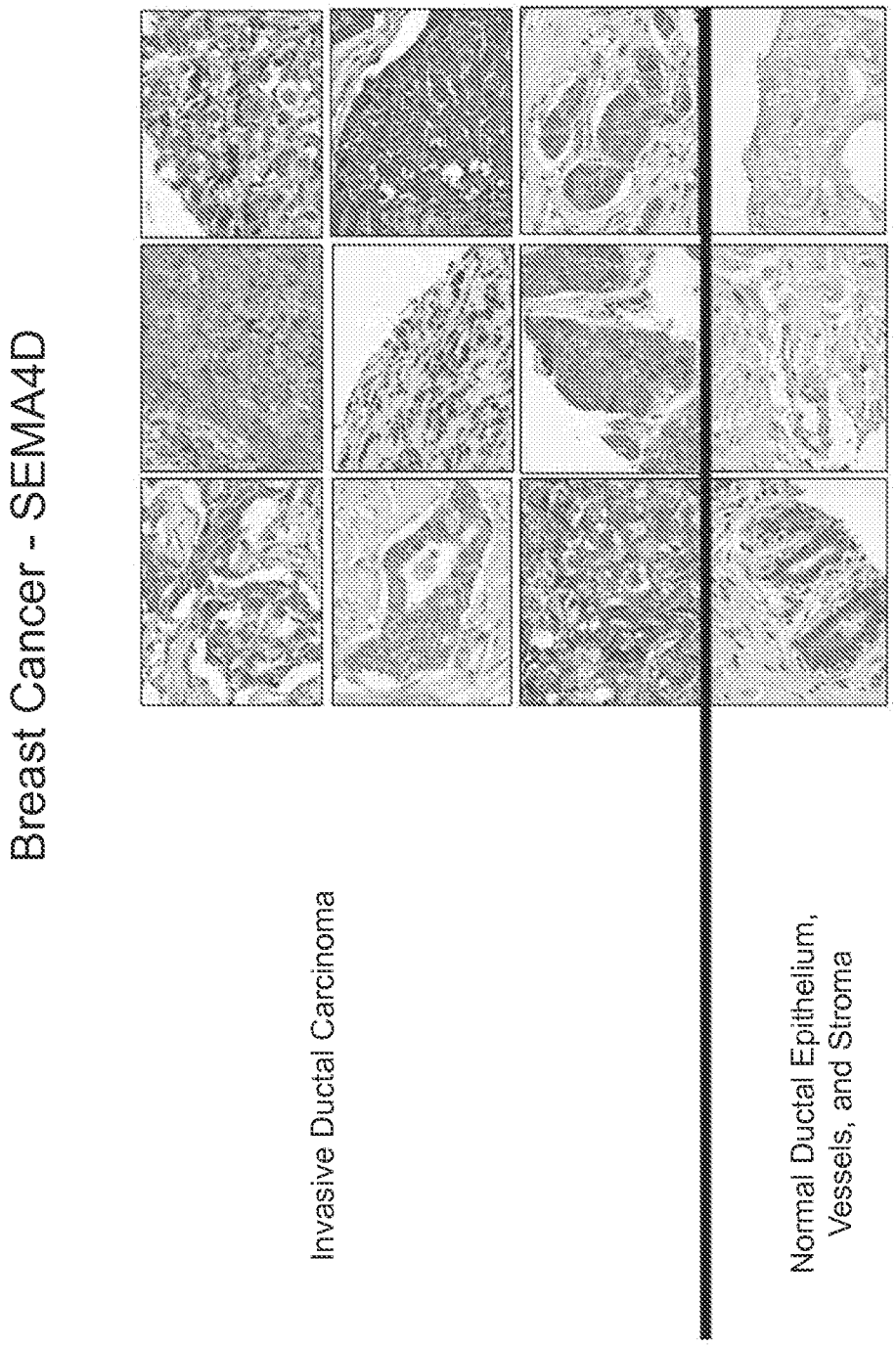
Breast Cancer - SEMA4D
Invasive Ductal Carcinoma
Normal Ductal Epithelium, Vessels, and Stroma

FIG. 16

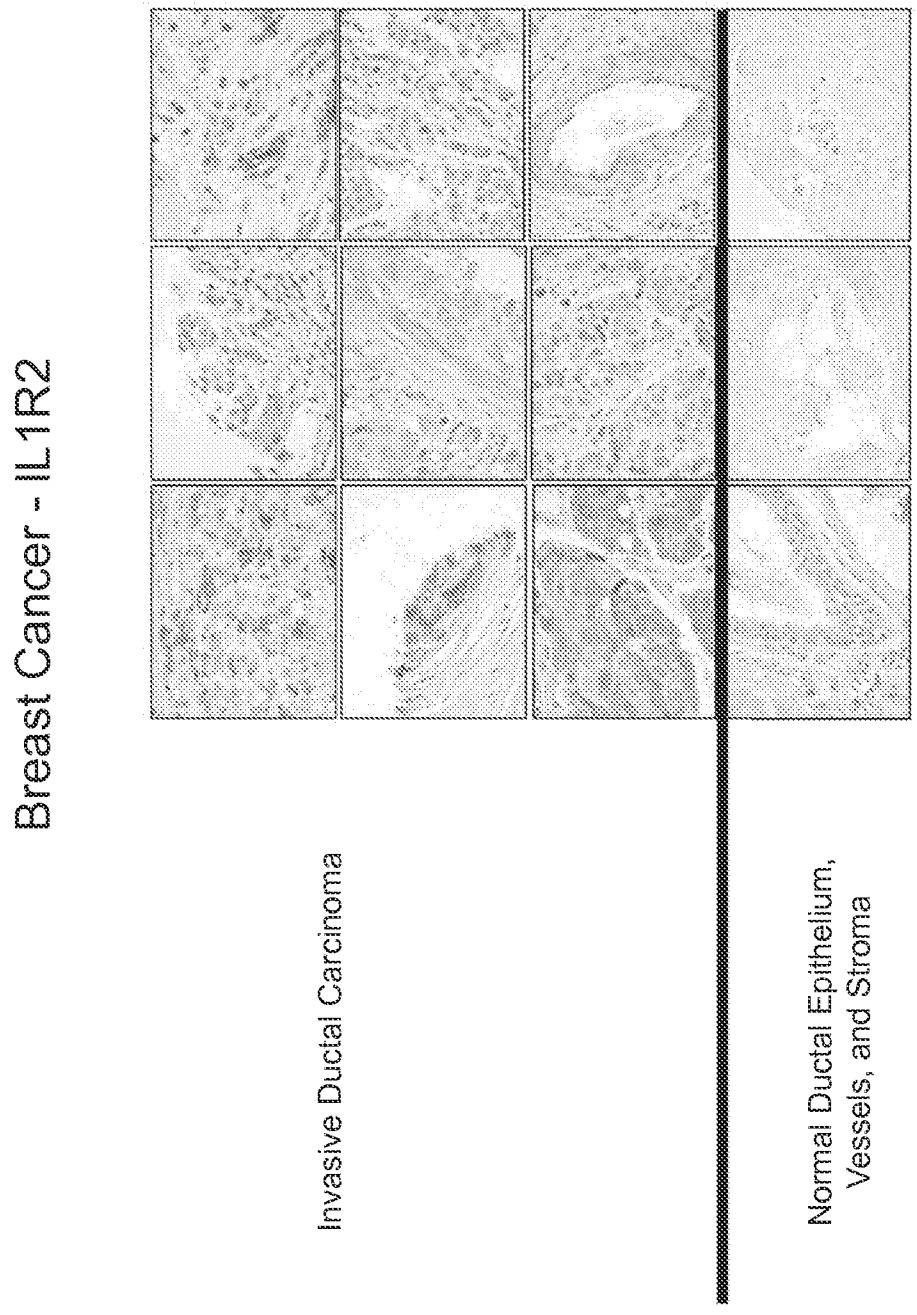
Breast Cancer - IL1R2
Invasive Ductal Carcinoma
Normal Ductal Epithelium, Vessels, and Stroma

FIG. 17

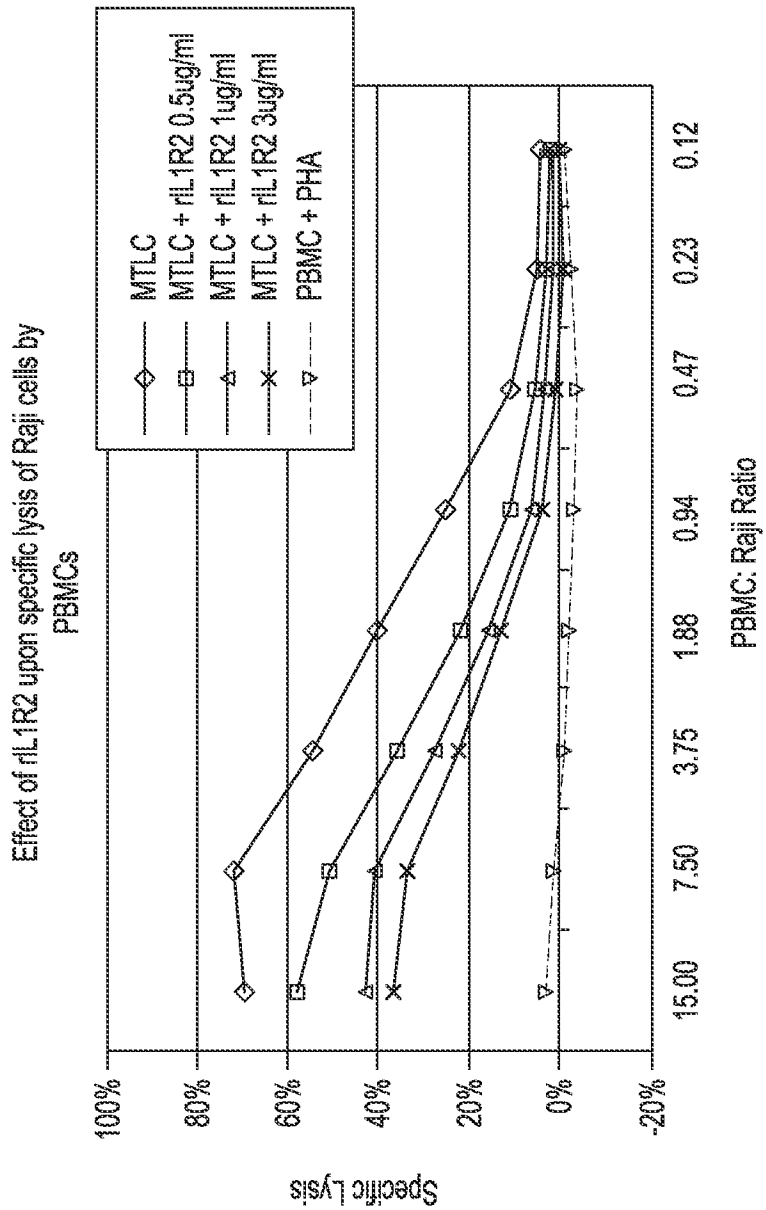
Effect of rIL1R2 upon specific lysis of Raji cells by PBMCs
MTLC
MTLC + rIL1R2 0.5ug/ml
MTLC + rIL1R2 1ug/ml
MTLC + rIL1R2 3ug/ml
PBMC + PHA
Specific Lysis
100%
80%
60%
40%
20%
0%
-20%
15.00
7.50
3.75
1.88
0.94
0.47
0.23
0.12
PBMC: Raji Ratio

FIG. 18

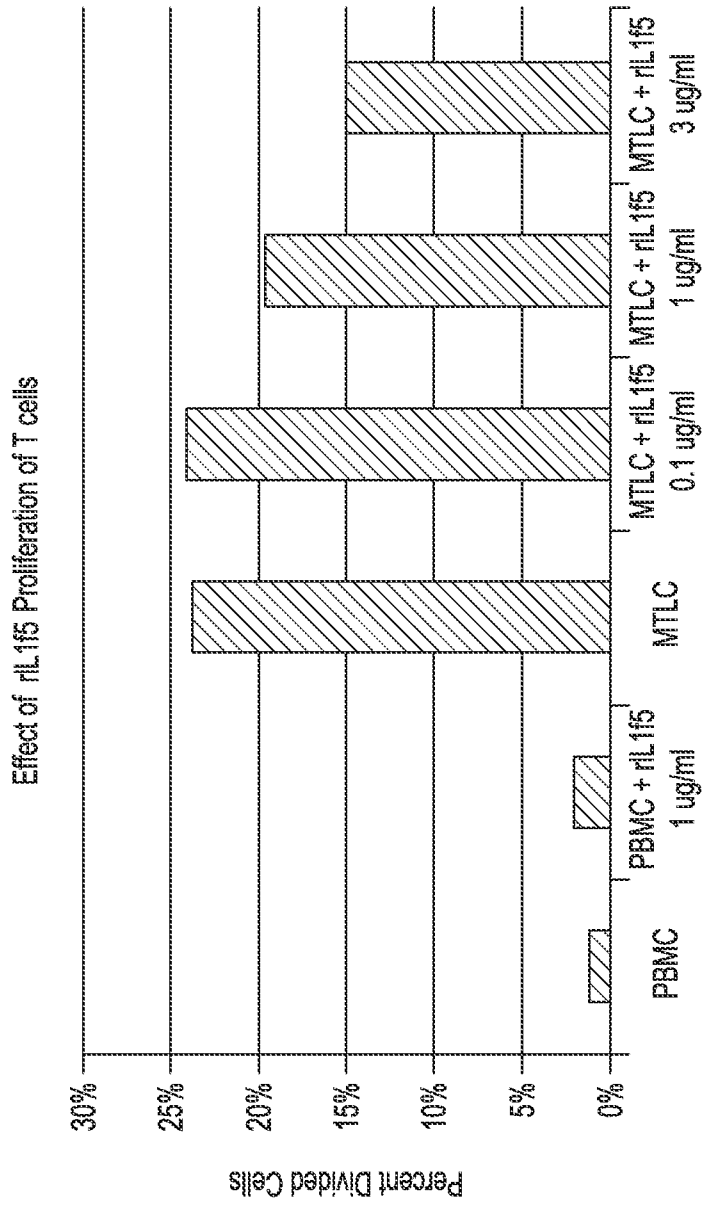
Effect of rIL1f5 Proliferation of T cells
Percent Divided Cells
30%
25%
20%
15%
10%
5%
0%
PBMC
PBMC + rIL1f5 1 ug/ml
MTLC
MTLC + rIL1f5 0.1 ug/ml
MTLC + rIL1f5 1 ug/ml
MTLC + rIL1f5 3 ug/ml

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/043080

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/20 A61K38/19 G01N33/50 A61K39/395
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002/187122 A1 (SIMS JOHN E [US]) 12 December 2002 (2002-12-12) page 17, left-hand column, paragraph 202 - right-hand column, paragraph 205	2
X	US 5 945 310 A (YOUNG PETER RONALD [US] ET AL) 31 August 1999 (1999-08-31) column 14, last paragraph - column 15, line 20	2
X	WO 02/00690 A2 (GENENTECH INC [US]; BAKER KEVIN P [US]; FERRARA NAPOLEONE [US]; GERBER) 3 January 2002 (2002-01-03) claims 20, 23, 27, 29, 30; sequence 316	1-30

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 July 2012	26/11/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/043080

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A. JOHNSTON ET AL: "IL-1F5, -F6, -F8, and -F9: A Novel IL-1 Family Signaling System That Is Active in Psoriasis and Promotes Keratinocyte Antimicrobial Peptide Expression", THE JOURNAL OF IMMUNOLOGY, vol. 186, no. 4, 17 January 2011 (2011-01-17), pages 2613-2622, XP055033275, ISSN: 0022-1767, DOI: 10.4049/jimmunol.1003162 the whole document	1-30
A	MULERO J J ET AL: "Organization of the human interleukin -1 receptor antagonist gene IL1HY1", IMMUNOGENETICS, SPRINGER VERLAG, BERLIN, DE, vol. 51, 1 May 2000 (2000-05-01), pages 425-428, XP002217818, ISSN: 0093-7711, DOI: 10.1007/S002510050640 the whole document	1-30
A	WO 2007/034465 A2 (TRINITY COLLEGE DUBLIN [IE]; MILLS KINGSTON [IE]; LYNCH MARINA [IE]; C) 29 March 2007 (2007-03-29) paragraph [0188] paragraph [0219]	1-30
A,P	HAL BLUMBERG ET AL: "Opposing activities of two novel members of the IL-1 ligand family regulate skin infl ammation", THE JOURNAL OF EXPERIMENTAL MEDICINE, ROCKEFELLER UNIVERSITY PRESS, US, vol. 204, no. 11, 1 January 2614 (2614-01-01), pages 20071029-2603, XP007911379, ISSN: 0022-1007	1-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/043080

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-30(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-30(partially)

Use of IL1F5 (=IL36RN, IL-1-delta, IL1HY1, IL-36RA, FIL1 delta, IL1RP3, PSORP, MGC29840, IL-1RP) protein (according to SeqID 1) , polynucleotides, complementary oligonucleotides or Ab thereto for use in the treatment and diagnosis of cancer

---

2-6. claims: 1-30(partially)

Use of CCBP2 (SeqID 2) / IL1R2 (SeqID 3) / IL1RAPL1 (SeqID 4) / IL18BP (SeqID 5) / CLEC2B (SeqID 6) protein, polynucleotides, complementary oligonucleotides or Ab thereto for use in the treatment and diagnosis of cancer.

7-9. claims: 1-30(partially)

Use of Serpin I1 (SeqID 9) / Serpin A4 (SeqID 18) / Serpin B5 (SeqID 19) protein, polynucleotides, complementary oligonucleotides or Ab thereto for use in the treatment and diagnosis of cancer.

---

10. claims: 1-30(partially)

Use of IL1RAP isoform 1 (SeqID 10) or 2 (SeqID 11) protein, polynucleotides, complementary oligonucleotides or Ab thereto for use in the treatment and diagnosis of cancer.

11-16. claims: 1-30(partially)

Use of GPR1 (SeqID 12) / GPR4 (SeqID 13) / GPR15 (SeqID 14) / GPR32 (SeqID 15) / GPR34 (SeqID 16) / GPR183 (SeqID 17) protein, polynucleotides, complementary oligonucleotides or Ab thereto for use in the treatment and diagnosis of cancer.

---

17. claims: 1-30(partially)

Use of Sema 4B (SeqID 20) or Sema 4D (SeqID 21) protein, polynucleotides, complementary oligonucleotides or Ab thereto for use in the treatment and diagnosis of cancer.

18-20. claims: 1-30(partially)

Use of CCL14 (SeqID 22) / NKTR (SeqID 23) / SFTPD (SeqID 24) protein, polynucleotides, complementary oligonucleotides or Ab thereto for use in the treatment and diagnosis of

cancer.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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