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(54) Title: A METHOD FOR THE TREATMENT OF MALIGNANT DISEASES BY INHIBITING NUCLEOLIN

(57) Abstract: Use for anti-nucleolin agents, such as antibodies, antisense molecules and interfering RNA molecules, in the treatment of cancers. The treatment is preferably combined with the administration of a chemotoxic or chemotherapeutic agent, or with radiation therapy.

A METHOD FOR THE TREATMENT OF MALIGNANT DISEASES BY
INHIBITING NUCLEOLIN

BACKGROUND

Cancer

Being well-prepared for battle engenders success; when the foe is cancer, early detection results in a greater likelihood that medical intervention will be successful. At early stages, treatments can often be targeted only to the affected tissues, diminishing side effects. If not caught early, cancer cells may metastasize and spread throughout the body. The prognosis in this case is more dire, and medical treatments are often applied systemically, killing not only cancer cells, but large numbers of healthy cells.

The National Cancer Institute estimates that in 2002, 1,285 million Americans will be newly diagnosed with cancer, and more than 560,000 Americans will die from cancer related illness.

A hallmark of a cancer cell is uncontrolled proliferation. Uncontrolled proliferation of these cells can manifest as cell masses (tumors) that interfere with normal organ function. If proliferation is not controlled or contained, cells from tumors migrate and colonize other tissues of the body, eventually resulting in death.

External factors, such as tobacco smoke, radiation and viruses, can lead to alterations in specific genes that result in unregulated cellular proliferation. Intrinsic factors, including inheritable mutations, hormone levels and metabolism, contribute to one's risk of contracting cancer.

Cancer cells also exhibit morphological and functional aberrations. Cellular morphology may be less organized; for example, the cells losing the asymmetric

organelle and structural organization (cell polarity) that allows for proper cell function. Cell-cell and cell-substratum contacts, the specificities of which are also necessary for normal function, are often modulated or lost. Functionally, the cells may carry on few, if any, wild-type functions, or may have exaggerated, unregulated normal functions, such as hormone secretion. Such cells regress to early developmental stages, appearing less differentiated than their wild-type (*i.e.*, normal) parents.

10 Cancer cells also often mis-express or mis-target proteins to inappropriate cellular compartments. Proteins may be up- or down-regulated; even proteins not usually expressed by a specific cell type can be expressed by the transformed counterpart. Protein mis-expression can have a plethora of downstream cellular effects, including drastic changes in membrane composition, organelle formation, or physiology. Mis-targeting of proteins (and other molecules, such as lipids, *etc.*) also contributes to the loss of cell polarity.

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Treatments for cancer

Methods for treating cancers include surgery (physical removal of the cancerous tissues), radiation therapy (killing cells by exposure to cell-lethal doses of radioactivity), chemotherapy (administering chemical toxins to the cells), immunotherapy (using antibodies that target cancer cells and mark them for destruction by the innate immune system) and nucleic acid-based therapies (e.g., expression of genetic material to inhibit cancer growth). Each approach, however, has its limitations.

25 Surgery, chemotherapy and radiation therapy suffer from similar significant limitations, such as incomplete removal of cancer cells or the inadvertent killing of healthy cells. Surgical tactics are most effective when the cancers are in early stages and limited localized area in the body. Even in the few cases that are diagnosed early, surgical removal of cancerous cells is often incomplete, and re-emergence of metastatic lesions often follow. When chemotherapeutic agents are administered in precise doses, they are preferentially toxic to rapidly proliferating cancer cells and not injuring the majority of healthy cells. Locally delivered doses of external beam 30 radiation are most effective on rapidly growing cells, killing them by introducing non-

specific, DNA-damage. Radiation therapy, like surgery, works best when the targeted cancer mass is well delimited; the balance of killing healthy cells *versus* cancer cells must be carefully weighed. Both chemotherapy and radiation therapy are not entirely selective for cancerous cells; inevitably, some healthy cells fall victim to the toxic effects, inflicting profound side-effects on the already-suffering cancer victim.

5 Other common approaches, immunotherapy and gene therapy, can be quite powerful and surpass surgery, chemo- and radiation therapy. These techniques target specific factors that are associated with tumor survival, cell growth or metastasis. For example, antibodies can target specific tumor-associated proteins, such as the monoclonal antibody that binds to a surface protein specific to the B-cells, CD20 (RITUXAN®; Genentech, Inc. and IDEC Inc.) that is used to treat B-cell malignancies. 10 An example of an effective gene therapy is anti-sense inhibition of *bcl-2* expression (GENASENSE®; Genta, Inc.). While effective, the challenge is to identify those 15 clinically relevant genes and proteins and develop appropriate therapeutics that target them to result in the destruction of the cancer cell. Furthermore, the process is not only laborious in identifying these molecules, but in many instances, the identified molecules will be specific to only one type of cancer or tumor cell.

20 All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that 25 any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

SUMMARY

A first aspect provides a method of treating cancer in a mammal comprising 30 administering to said mammal a therapeutically effective amount of an anti-nucleolin agent and a pharmaceutically acceptable carrier, wherein the anti-nucleolin agent comprises an anti-nucleolin antibody.

A second aspect provides use of an anti-nucleolin agent in the manufacture of a medicament for treating cancer in a mammal, wherein the anti-nucleolin agent comprises an anti-nucleolin antibody.

Disclosed herein are methods for treating tumors or cancer in a subject by:

- 5 (1) administering a therapeutically effective amount of an anti-nucleolin agent and a pharmaceutically acceptable carrier. This pharmaceutical composition may further comprise other chemotherapeutic or chemotoxic agents, such as cyclophosphamide, etoposide, doxorubicin, methotrexate, vincristine, procabazine, prednizone, dexamethasone, tamoxifen citrate, carboplatin, cisplatin, oxaliplatin, 5-fluorouracil, camptothecin, zoledronic acid, ibandronate and mytomycin. In conjunction, radiation therapy may also be applied.
- 10 (2) administering a therapeutically effective amount of an anti-nucleolin antibody and a pharmaceutically acceptable carrier, wherein the antibody is substantially non-immunogenic to human. This pharmaceutical composition may further comprise other chemotherapeutic or chemotoxic agents. In conjunction, radiation therapy may also be applied.
- 15 (3) administering a therapeutically effective amount of an anti-nucleolin antibody, a chemotoxic or chemotherapeutic agent and a pharmaceutically acceptable carrier.
- 20 (4) administering a therapeutically effective amount of a nucleolin antibody and a pharmaceutically acceptable carrier, and further treating the subject with radiation therapy.
- (5) administering a therapeutically effective amount of a duplex interfering RNA to nucleolin and a pharmaceutically acceptable carrier.
- 25 (6) administering a nucleolin anti-sense molecule which inhibits the production of the nucleolin protein.
- (7) administering a nucleolin-interfering RNA molecule which inhibits the expression of the nucleolin gene.

In the first or second aspects, a chemotherapeutic or chemotoxic agent may be cyclophosphamide, etoposide, doxorubicin, methotrexate, vincristine, procabazine, prednizone, dexamethasone, tamoxifen citrate, carboplatin, cisplatin, oxaliplatin, 5-fluorouracil, camptothecin, zoledronic acid, ibandronate and mytomycin.

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Also disclosed are pharmaceutical compositions that comprise a pharmaceutically acceptable carrier and:

5 (8) an anti-sense oligonucleotide directed against nucleolin.
(9) an inhibitory RNA against nucleolin.
(10) a nucleolin antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows nuclear nucleolin staining in various cell lines. Shown are immunofluorescent (B, D, F, H) and parallel phase contrast micrographs (A, C, E, G). Cell lines that were analyzed were: DU145 prostate cancer cells (A, B), MDA-MB-231 breast cancer cells (C, D), HeLa cervical cancer cells (E, F) and HS27 normal skin cells (G, H). An anti-nucleolin antibody was used; the cells were permeabilized

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before staining to allow the antibody access to the cytoplasmic and nuclear compartments.

5 FIG. 2 shows plasma membrane nucleolin staining in the cell lines shown in Figure 1. Shown are immunofluorescent (B, D, F, H) and parallel phase contrast micrographs (A, C, E, G). Cell lines that were analyzed were: DU145 prostate cancer cells (A, B), MDA-MB-231 breast cancer cells (C, D), HeLa cervical cancer cells (E, F) and HS27 normal skin cells (G, H). An anti-nucleolin antibody was used; the cells were not permeabilized before staining, allowing the antibody access to only 10 the plasma membrane.

15 FIG. 3 shows the comparative proliferation rates of cell lines as measured by MTT assay. Square, DU145; diamonds, HeLa; circles, HS27. Although MDA-MB-231 was not included in this experiment, proliferation rates for these four cell lines have been determined to be DU145 > MDA-MB-231 > HeLa > HS27. Note that the cell lines with high levels of plasma membrane nucleolin correspond to those with the most rapid proliferation (DU145 and HeLa; see Figure 2).

20 FIG. 4 shows phase contrast (B,D) and immunofluorescent image (A,C) of a paraffin-embedded specimen resected from a patient with squamous cell carcinoma of the head and neck. The specimen was stained for plasma membrane nucleolin and counter-stained with propidium iodide to show cell nuclei. Images (C) and (D) show 25 images (A) and (B) overlaid with markings to better show nucleolin staining. The area 1 encompassed by the white line includes intense nucleolin staining, while the areas outside of 1 show little 3 or no 2 signal.

30 FIG. 5 shows phase contrast (B, D) and immunofluorescent images (A, C) of small cell (NCI-H82) and non-small cell lung (NCI-H1299) cancer cell lines placed onto a microscope slide using a cytopinner. Samples were stained for plasma membrane nucleolin and counter-stained with propidium iodide to show cell nuclei. Cells with exceptionally well-stained plasma membranes are denoted by asterisks (*).

FIG. 6 shows phase contrast (B, D, F) and immunofluorescent images (A, C, E) of peripheral blood (A, B) or bone marrow (C, D and E, F) from human subjects. Samples were stained for plasma membrane nucleolin and counter-stained with propidium iodide to show cell nuclei. Highly stained cells for nucleolin are marked with an asterisk (*); these were only seen in those patients suffering from carcinomas (A,B and C,D), while cells from a healthy patient did not display any plasma membrane nucleolin staining (E, F).

DETAILED DESCRIPTION

The invention is based on the discovery of a correlation between nucleolin plasma membrane expression and the presence and aggressiveness of neoplastic cells. The unexpected discovery that nucleolin, mostly restricted to the interior of the healthy cell nucleus, when found on the cell surface, correlates with a pre-malignant or malignant phenotype. Not only does this observation facilitate cancer diagnosis and prognosis, but also provides a novel and powerful treatment strategy. Disclosed herein are methods of treating cancer by administering a compound that specifically targets nucleolin. Furthermore, disclosed herein are methods for treating cancer by administering a nucleolin-targeting compound in conjunction with other cancer therapies, *e.g.*, an anti-cancer drug. Such combination therapy achieves superior and synergistic therapeutic results.

The advantages of using surface-localized nucleolin to treat tumors and cancers include:

- (1) Specificity. Plasma membrane nucleolin is not usually observed in the plasma membrane of most wild-type (healthy) cells. Thus, unlike other non-specific therapeutic approaches (*e.g.*, surgery, radiation, chemotoxins), plasma membrane nucleolin targeting can be used to specifically kill cancer cells.
- (2) Broad applicability. Unlike previous immuno- and gene therapies, nucleolin expression on the plasma membrane occurs on many types of cancer cells. Many different cancers, therefore, may be treated by exploiting plasma membrane

nucleolin; yet, unlike other less-specific treatments (e.g., radiation therapy), healthy cells are not damaged or killed.

5 (3) Treatment: early or late. Because plasma membrane nucleolin is indicative of not only malignant cells, but also of *pre*-malignant cells, treatment can commence with the detection of surface nucleolin, even before a tumor mass would usually be detected by other means.

10 While investigating the anti-proliferative activity of non-anti-sense guanosine-rich oligonucleotides (GROs) on cancer cells, it was found that such anti-proliferative GROs bind nucleolin to exert their effects (Bates *et al.*, 1999; Miller *et al.*, 2000).

15 Nucleolin (Bandman *et al.*, 1999) is an abundant, non-ribosomal protein of the nucleolus, the site of ribosomal gene transcription and packaging of pre-ribosomal RNA. This 707 amino acid phosphoprotein has a multi-domain structure consisting of a histone-like N-terminus, a central domain containing four RNA recognition motifs and a glycine/arginine-rich C-terminus and has an apparent molecular weight of 110 kD. Its multiple domain structure reflects the remarkably diverse functions of this multifaceted protein (Ginisty *et al.*, 1999; Srivastava and Pollard, 1999; Tuteja and Tuteja, 1998). Nucleolin has been implicated in many fundamental aspects of cell survival and proliferation. Most understood is the role of nucleolin in ribosome biogenesis. Other functions may include nucleocytoplasmic transport, cytokinesis, nucleogenesis and apoptosis. Nucleolin is one of the nuclear organizer region (NOR) proteins whose levels, as measured by silver staining, are assessed by pathologists as a marker of cell proliferation and an indicator of malignancy (Derenzini, 2000).

20 Also present in the cell plasma membrane in a few cell types, such as lymphocytes and inner medullary collecting duct cells, nucleolin has been hypothesized to function as a receptor (e.g., (Callebaut *et al.*, 1998; Sorokina and Kleinman, 1999). However, the role of plasma membrane nucleolin is not well understood. In addition, it is not clear whether the plasma membrane nucleolin is identical to the nucleolar protein, or if it represents a different isoform or nucleolin-like protein. However, the expression of plasma membrane nucleolin is specific to neoplastic cells (such as malignant or pre-malignant); thus the function of plasma membrane nucleolin need not be known for effective therapeutic intervention.

Definitions*neoplasm, malignancy, tumor, cancer cells*

5 A neoplasm is an abnormal tissue growth resulting from neoplastic cells, cells that proliferate more rapidly and uncontrollably than normal cells. Usually partially or completely structurally disorganized, neoplasms lack functional coordination with the corresponding normal tissue. Neoplasms usually form a distinct tissue mass that may be either benign (tumor) or malignant (cancer).

10 Cancer cells invade surrounding tissues, may metastasize to distant sites, and are likely to recur after attempted removal, causing death of a subject if not adequately treated. In addition to structural disorganization, cancer cells usually regress to more primitive or undifferentiated states (anaplasia), although morphologically and biochemically, they may still exhibit many functions of the corresponding wild-type cells. Carcinomas are cancers derived from epithelia; sarcomas are derived from connective tissues. In some cases, cancers may not be associated with a tumor, but like the affected tissue, is diffuse, e.g., leukemias.

15 Cancers may be more aggressive or less aggressive. The aggressive phenotype of a cancer cell refers to the proliferation rate and the ability to form tumors and metastasize in nude mice. Aggressive cancers proliferate more quickly, more easily form tumors and metastasize than less-aggressive tumors.

20 Tumors and cancers include solid, dysproliferative tissue changes and diffuse tumors. In the sense that cancers and tumors are abnormal growths having uncontrolled proliferation of cells that do not serve a normal physiological function, the terms "tumor" and "cancer" are used interchangeably. Examples of tumors and cancers include melanoma, lymphoma, plasmacytoma, sarcoma, glioma, thymoma, leukemia, breast cancer, prostate cancer, colon cancer, liver cancer, esophageal cancer, brain cancer, lung cancer, ovary cancer, cervical cancer, hepatoma, and other neoplasms. For more examples of tumors and cancers, see, for example (Stedman, 2000).

25 "Stromal cells" are accessory cells found within a tumor. Such cells may be, for example, fibroblasts, reticular cells and endothelial cells, and play a supportive role in tumor growth but are healthy cells. Stromal cells and fibroblasts therefore are constituents of the micro-environment in which tumor cells invade during metastasis.

neoplastic state

The term "neoplastic state" refers to three conditions: normal, pre-malignant and malignant. "Normal" refers to a growth or cell that is clinically normal (healthy). "Pre-malignant" refers to a growth or cell that is on the pathway to malignancy, but at the time of examination, would not be classified as malignant by conventional methods. "Malignant" refers to a cell or growth that has at least one of the following properties: locally invasive, destructive growth and metastasis.

GROs and other polypeptide-binding oligonucleotides

Oligonucleotides are available that specifically bind to polypeptides, such as nucleolins. Examples of such are GROs, which are guanosine-rich oligonucleotides. Characteristics of GROs include:

- (1) having at least 1 GGT motif
- (2) preferably having 4-100 nucleotides, although GROs having many more nucleotides are possible
- (3) having chemical modifications to improve stability.

Especially useful GROs form G-quartet structures, as indicated by a reversible thermal denaturation/renaturation profile at 295 nm (Bates *et al.*, 1999). Preferred GROs also compete with a telomere oligonucleotide for binding to a target cellular protein in an electrophoretic mobility shift assay (Bates *et al.*, 1999).

Other oligonucleotides may have high binding specificity for nucleolin.

anti-nucleolin agent

An "anti-nucleolin agent" binds to nucleolin. Examples include anti-nucleolin antibodies and certain oligonucleotides.

Nucleic acid-based definitions

A "structural gene" or "gene" refers to a DNA sequence that is transcribed into messenger RNA (mRNA) which can be translated into a polypeptide (a polypeptide consists of at least two amino acid residues).

A promoter is a DNA sequence that specifies the site of initiation of RNA transcription, the direction of transcription, and the rate of transcription. For this reason, promoters are usually located 5' of the start site (designated as +1) for the

5 DNA sequence that encodes the resultant RNA transcript. Promoters can be unregulated or regulated. When a promoter is unregulated, it operates constitutively at a particular basal level of activity. When the promoter is regulated, the efficiency of a promoter can be modulated in response to an agent. RNA transcription is increased relative to the basal transcription level under circumstances where an agent 10 positively regulates promoter activity; conversely, RNA transcription is decreased relative to the basal transcription level under circumstances where an agent negatively regulates promoter activity. Agents that positively regulate promoter activity are called activators; whereas agents that negatively regulate promoter activity are called repressors.

15 An enhancer is a DNA transcription element that can increase the efficiency of promoter activity. Like promoters, enhancers are physically linked to the affected gene. Enhancers may also be unregulated or regulated. Unlike promoters, however, enhancers cannot specify the start site of RNA transcription or the direction of transcription. Enhancers can stimulate gene expression independent of the enhancer's orientation and location with respect to the start site of RNA transcription. Because 20 enhancers do not specify the start site of RNA transcription, enhancers can exert their effects over great distances (several kilobases) with respect to a particular gene.

25 A regulatory sequence is typically a short DNA motif that positively or negatively responds to the activity of an agent. A regulatory sequence may be bidirectional or unidirectional. A regulatory sequence can be part of the modular organization of either a promoter or an enhancer. In the context of a promoter, a regulatory sequence either modulates promoter efficiency and/or affects the selection of initiation sites of RNA transcription. In the context of an enhancer, a regulatory sequence modulates the efficiency of an enhancer.

30 A "cloning vector" is a DNA molecule such as a plasmid, cosmid, or bacteriophage that has the ability to replicate in a cell. Cloning vectors typically contain restriction endonuclease recognition sites that enable the introduction of changes and additions of DNA fragments. Cloning vectors also typically include promoters to enable efficient expression and selectable markers that confer resistance to compounds such as ampicillin or tetracycline.

An "expression vector" is a polynucleotide comprising a coding sequence (such as a gene) that is made to be expressed by the host cell. Expression vectors typically contain promoters, enhancers and tissue specific regulatory elements that are operably linked to the expressed gene or DNA fragment.

5 The term "isoform" refers to polypeptides that differ in amino acid sequence or post-translational modifications (such as glycosylation or proteolytic processing events). Isoforms are also used to refer to polypeptides arising from a common gene which result from alternative splicing.

10 *Therapy-related definitions*

"Cytotoxic agent" refers to a substance that inhibits or prevents at least one function of a cell, or causes the destruction of a cell. Radioactive isotopes (e.g., ^{211}At , ^{90}Y , ^{186}Re , ^{188}Re , ^{153}Sm , ^{212}Bi , ^{32}P and radioactive isotopes of Lu), and chemotherapeutic agents or toxins, such as small molecule toxins, or toxins from bacteria, fungi, plants or animals are examples of such agents.

"Radiation therapy" and "radiotherapy" refers to the use of locally delivered doses of external beam radiation to effect killing of a tumor or cancer cell.

A “chemotherapeutic agent” is a chemical compound that can be used effectively to treat a cancer cell. Examples of commonly used oncology drugs and agents include vinorelbine (Navelbine®), mytomycin, camptothecin, cyclophosphamide (Cytoxin®), methotrexate, tamoxifen citrate, 5-fluorouracil, irinotecan, doxorubicin, flutamide, paclitaxel (Taxol®), docetaxel, vinblastine, imatinib mesylate (Gleevec®), anthracycline, letrozole, arsenic trioxide (Trisenox®), anastrozole, triptorelin pamoate, ozogamicin, irinotecan hydrochloride (Camptosar®), BCG, live (Pacis®), leuprolide acetate implant (Viadur), bexarotene (Targretin®), exemestane (Aromasin®), topotecan hydrochloride (Hycamtin®), gemcitabine HCL (Gemzar®), daunorubicin hydrochloride (Daunorubicin HCL®), gemcitabine HCL (Gemzar®), toremifene citrate (Fareston), carboplatin (Paraplatin®), cisplatin (Platinol® and Platinol-AQ®) oxaliplatin and any other platinum-containing oncology drug.

"Medicament," "therapeutic composition" and "pharmaceutical composition" are used interchangeably to indicate a compound, matter, mixture or preparation that exerts a therapeutic effect in a subject.

5 "Approved therapeutic antibodies" include rituximab (Rituxan®), gemtuzumab (Mylotarg®), alemtuzumab (Campath®) and trastuzumab (Herceptin®).

"Antibody" is used in the broadest sense and refers to monoclonal antibodies, polyclonal antibodies, multispecific antibodies, antibody fragments and derivatives.

An "artificial antibody" is a binding agent having polypeptide binding domains connected to polypeptide scaffolds (Irving *et al.*, 2001; Koide, 2002).

10 A "conjugated antibody drug" refers to a therapeutic agent which includes an antibody or antibody fragment which is conjugated to a moiety that is a cytotoxic agent.

An "antibody fusion protein" refers to a recombinant molecule that comprises one or more antibody components and a therapeutic agent such as a cytokine, enzyme or a cytotoxic agent.

15 The term "naked antibody" refers to an entire, intact antibody, such as a monoclonal, recombinant monoclonal or polyclonal antibody that is neither fused nor conjugated to an enzyme, cytotoxic agent or chemotherapeutic agent.

20 A "nucleolin antibody" or "anti-nucleolin antibody" is an antibody, conjugated antibody drug, antibody fusion protein, naked antibody or artificial antibody that binds to nucleolin polypeptides.

"Anti-sense oligonucleotides," "oligo," "oligo nucleic acid," "anti-sense," or "anti-sense polynucleotide" are sequence-specific drugs capable of selectively modifying or silencing the expression of genes, causing a desired therapeutic effect.

25 The term "interfering RNA," "RNAi," "short interfering RNA" or "double stranded interfering RNA" refer to either free ribonucleic acid molecules or those generated *in vivo* by means of gene expression systems. Such interfering RNA molecules are able to alter the expression of a target gene.

30 "Treating a tumor" or "treating a cancer" means to significantly inhibit tumor/cancer growth and/or metastasis. Growth inhibition can be indicated by reduced tumor volume or reduced occurrences of metastasis. Tumor growth can be determined, *e.g.*, by examining the tumor volume *via* routine procedures (such as

obtaining two-dimensional measurements with a dial caliper). Metastasis can be determined by inspecting for tumor cells in secondary sites or examining the metastatic potential of biopsied tumor cells *in vitro* using well-known techniques.

5 An "anti-nucleolin agent" includes any molecule, compound, *etc.*, that interacts with nucleolin. Such agents include anti-nucleolin antibodies and derivatives thereof, anti-sense oligonucleotides, ribozymes, RNAi, *etc.*

10 In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

Embodiments

15 The following embodiments are given as examples of various ways to practise the invention. Many different ways of practising the invention are also possible.

20 In all embodiments, the underlying principle is to target cells for therapeutic invention by specifically differentiating between plasma membrane nucleolin and nuclear nucleolin. Plasma membrane nucleolin, as discovered by the applicants, correlates with cells that are in a neoplastic state. Exploiting this differential plasma membrane expression, tumor and cancer cells can be targeted for treatment.

Detection of plasma membrane nucleolin

25 Various techniques allow a user to differentiate between nuclear and plasma membrane nucleolin. Detection techniques, wherein the nucleolin-detecting reagents have exclusive access to extracellular portions of the cell (and consequently cell-plasma membrane nucleolin), or biochemical techniques, wherein either the surface plasma membrane and/or surface proteins are separated from other cellular components and compartments, are also useful. One practising the invention may wish to determine the potential effectiveness of a therapy targeting nucleolin by first examining the cells for 30 nucleolin plasma membrane expression.

In an embodiment, nucleolin is detected directly on the cell surface. A cell is isolated from a subject and plasma membrane nucleolin detected using an agent that

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binds nucleolin. Cells may be isolated by any known technique. An isolated cell may comprise a larger tissue sample containing cells that are not neoplastic. Detection procedures use anti-nucleolin antibodies that bind extracellular nucleolin epitopes; these antibodies may be directly labeled or when bound, detected indirectly. Other useful plasma membrane nucleolin detection agents include GROs that specifically

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bind nucleolin. Useful procedures, such as fluorescence-activated cell sorting (FACS) or immunofluorescence, employ fluorescent labels, while other cytological techniques, such as histochemical, immunohistochemical and other microscopic (electron microscopy (EM), immuno-EM) techniques use various other labels, either 5 colorimetric or radioactive. The various reagents may be assembled into kits.

In another embodiment, cells are isolated from a subject and extracted. Plasma membranes and/or proteins are then isolated (such as *via* differential extraction, or differential physical cell disruption, differential centrifugation of detergent-extracted cells, *etc.*), and then nucleolin detected in the isolated membranes 10 using an agent that binds nucleolin. In general, useful techniques to detect nucleolin include those wherein the extract is placed on a substrate, and the substrate probed with a nucleolin-detecting reagent. Examples of such techniques include polypeptide dot blots and Western blots, biochips, protein arrays, *etc.*. Other detection formats include enzyme-linked immunosorbent assays (ELISAs) in their manifold 15 manifestations (Ausubel *et al.*, 1987). In embodiments wherein plasma membrane surface molecules are physically separated from most of the other cellular components and compartments, the nucleolin-binding agents need not specifically recognize any extracellular portions of nucleolin. The various reagents may be assembled into kits.

20 In a further embodiment, the methods of the invention are directed to detecting lung cancer, such as lung small cell carcinomas. Plasma membrane nucleolin expression is useful for detection and prognosis.

In one embodiment, the invention provides a method for the identification of a 25 subject for whom a certain therapy, such as nucleolin-directed chemotherapy, is indicated for the treatment or amelioration of a condition associated with an increased abundance of nucleolin on the cell surface. Such a condition includes cancer, neoplasia and a precancerous lesion. Such a method may include the steps of contacting an agent that binds specifically to nucleolin with a cell in or from a subject and determining the amount of binding of plasma membrane nucleolin.

30 In another embodiment, an individual who has, in the past, presented with cancer presents to a health-care provider for examination. A sample having a cell, such as a biopsy specimen, is taken from the individual's body, whereupon the sample

5 is contacted with an anti-nucleolin antibody, incubated, and the bound antibody then detected. The amount of bound antibody can be compared to the amount bound by healthy cells isolated from the same individual. A treatment regimen may then be devised, based on the quantity of cell-surface nucleolin per cell taken from the individual's body and a known correlation between cell-surface nucleolin and susceptibility of the cancer to a certain manner of therapy, such as chemotherapy targeting nucleolin.

10 *Treatment of tumor/cancer cells targeting nucleolin*
In one embodiment, methods of treating cells in a neoplastic state including cancer and tumor cells, are provided; these methods exploit plasma membrane nucleolin which acts as a beacon for a therapeutic agent. For example, administration of anti-nucleolin antibodies, which may be conjugated to a toxin or other means of stimulating cell death or incurring necrosis, results in the removal of plasma membrane nucleolin-expressing cells.

15

20 **Practicing the invention**
The following, not meant to limit the invention, is presented to aid the practitioner in carrying out the invention, although other methods, techniques, cells, reagents and approaches can be used to achieve the invention.

25 *Cells*
Cells or tissue samples are collected from a subject. The subject is a vertebrate, more preferably a mammal, such as a monkey, dog, cat, rabbit, cow, pig, goat, sheep, horse, rat, mouse, guinea pig, *etc.*; and most preferably a human. Any technique to collect the desired cells may be employed, including biopsy, surgery, scrape (inner cheek, skin, *etc.*) and blood withdrawal. Any appropriate tool may be used to carry out such tasks. It is not necessary to isolate the test population (*i.e.*, those cells being tested for neoplastic state) from those cells and tissues (contaminating material) that are not being tested, except in some cases using biochemical methods that include extraction. In this last case, the test population need not be completely isolated from contaminating materials, but should either

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predominate or be easily distinguishable (e.g., morphologically (structurally, specific markers) or biochemically).

For those methods that analyze lung carcinomas, sputum collection is an attractive and easily obtained sample. The term "sputum" as used herein refers to expectorated matter made up of saliva and discharges from the respiratory airways. Sputum is a highly complex material that has a pronounced gel-like structure.

For collection of sputum, Byrne, *et al.*, (Byrne, 1986) suggest that the patient collect material, raised by several deep coughs, in a container with a lid.

Alternatively, sputum can be collected by using a bronchoscope (Kim *et al.*, 1982).

Specific devices or agents may be used to facilitate sputum collection (Babkes *et al.*, 2001; King and Speert, 2002; Rubin and Newhouse, 1999). Other methods of sputum collection are also available.

Cell culture

In some cases, culturing the harvested cells is desirable to augment their numbers so that plasma membrane nucleolin detection is facilitated. Suitable media and conditions for generating primary cultures are well known. The selection of the media and culture conditions vary depending on cell type and may be empirically determined. For example, skeletal muscle, bone, neurons, skin, liver, and embryonic stem cells are grown in media that differs in their specific contents. Furthermore, media for one cell type may differ significantly from laboratory to laboratory and institution to institution. To keep cells dividing, serum, such as fetal calf serum (FCS) (also known as fetal bovine serum (FBS)), is added to the medium in relatively large quantities, 5%-30% by volume, depending on cell or tissue type. Other sera include newborn calf serum (NCS), bovine calf serum (BCS), adult bovine serum (ABS), horse serum (HS), human, chicken, goat, porcine, rabbit and sheep sera. Serum replacements may also be used, such as controlled process serum replacement-type (CPSR; 1 or 3) or bovine embryonic fluid. Specific purified growth factors or cocktails of multiple growth factors can also be added or sometimes substituted for serum. Specific factors or hormones that promote proliferation or cell survival can also be used.

Examples of suitable culture media include Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium Eagle (MEM), Basal Medium Eagle (BME), Click's Medium, L-15 Medium Leibovitz, McCoy's 5A Medium, Glasgow Minimum Essential Medium (GMEM), NCTC 109 Medium, Williams' Medium E, RPMI-1640, and Medium 199. A medium specifically developed for a particular cell type/line or cell function, e.g. Madin-Darby Bovine Kidney Growth Medium, Madin-Darby Bovine Kidney Maintenance Medium, various hybridoma media, Endothelial Basal Medium, Fibroblast Basal Medium, Keratinocyte Basal Medium, and Melanocyte Basal Medium are also known. If desired, a protein-reduced or -free and/or serum-free medium and/or chemically defined, animal component-free medium may be used, e.g., CHO, Gene Therapy Medium or QBSF Serum-free Medium (Sigma Chemical Co.; St. Louis, MO), DMEM Nutrient Mixture F-12 Ham, MCDB (105, 110, 131, 151, 153, 201 and 302), NCTC 135, Ultra DOMA PF or HL-1 (both from Biowhittaker; Walkersville, MD), may be used.

The medium can be supplemented with a variety of growth factors, cytokines, serum, etc., depending on the cells being cultured. Examples of suitable growth factors include: basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factors (TGF α and TGF β), platelet derived growth factors (PDGFs), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), insulin, erythropoietin (EPO), and colony stimulating factor (CSF). Examples of suitable hormone additives are estrogen, progesterone, testosterone or glucocorticoids such as dexamethasone. Examples of cytokine medium additives are interferons, interleukins or tumor necrosis factor- α (TNF α). Salt solutions may also be added to the media, including Alsever's Solution, Dulbecco's Phosphate Buffered Saline (DPBS), Earle's Balanced Salt Solution, Gey's Balanced Salt Solution (GBSS), Hanks' Balanced Salt Solution (HBSS), Puck's Saline A, and Tyrode's Salt Solution. If necessary, additives and culture components in different culture conditions be can optimized, as these may alter cell response, activity lifetime or other features affecting bioactivity. In addition, the surface on which the cells are grown can be coated with a variety of substrates that contribute to survival, growth and/or differentiation of the cells. These substrates

5 include but are not limited to, laminin, EHS-matrix, collagens, poly-L-lysine, poly-D-lysine, polyornithine and fibronectin. When three-dimensional cultures are desired, extracellular matrix gels may be used, such as collagen, EHS-matrix, or gelatin (denatured collagen). Cells may be grown on top of such matrices, or may be cast within the gels themselves.

10 If desired, the media may be further supplemented with reagents that limit acidosis of the cultures, such as buffer addition to the medium (such as N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (BIS-Tris), N-(20hydroxyethyl)piperazine-N'3-propanesulfonic acid (EPSS or HEPPS), glycylglycine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 3-(N-morpholino)propane sulfonic acid (MOPS), Piperazine-N,N'-bis(2-ethane-sulfonic acid) (PIPES), sodium bicarbonate, 3-(N-tris(hydroxymethyl)-methyl-amino)-2-hydroxy-propanesulfonic acid (TAPSO, (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), N-tris(hydroxymethyl)methyl-glycine (Tricine), tris(hydroxymethyl)-aminomethane (Tris), etc.). Frequent medium changes and changes in the supplied CO₂ (often approximately 5%) concentration may also be used to control acidosis.

15 20 25 Gases for culture typically are about 5% carbon dioxide and the remainder nitrogen, but optionally may contain varying mounts of nitric oxide (starting as low as 3 ppm), carbon monoxide and other gases, both inert and biologically active. Carbon dioxide concentrations typically range around 5%, but may vary between 2-10%. Both nitric oxide and carbon monoxide, when necessary, are typically administered in very small amounts (*i.e.* in the ppm range), determined empirically or from the literature. The temperature at which the cells will grow optimally can be empirically determined, although the culture temperature will usually be within the normal physiological range of the animal from which the cells were isolated.

Detecting nucleolin: antibody-based methods

Antibodies

30 Nucleolin can be detected at the protein level in cells, tissue sections, cultured cells and extracts thereof. Immunochemical methods to detect protein expression are well known and include, but are not limited to, Western blotting, immunoaffinity

purification, immunoprecipitation, enzyme-linked immunosorbent assay (ELISA), dot or slot blotting, radioimmunoassay (RIA), immunohistochemical detection, immunocytochemical staining, and flow cytometry. Common procedures and instructions using antibodies have been well addressed (e.g., (Harlow and Lane, 1988; Harlow and Lane, 1999). Selected antibodies that are useful for detecting plasma membrane nucleolin are shown in Table 1.

Table 1 Anti-nucleolin antibodies

Antibody	Source	Antigen source	Notes
p7-1A4 mouse monoclonal antibody (mAb)	Developmental Studies Hybridoma Bank (University of Iowa; Ames, IA)	<i>Xenopus laevis</i> oocytes	IgG ₁
sc-8031 mouse mAb	Santa Cruz Biotech (Santa Cruz, CA)	human	IgG ₁
sc-9893 goat polyclonal Ab (pAb)	Santa Cruz Biotech	human	IgG
sc-9892 goat pAb	Santa Cruz Biotech	human	IgG
clone 4E2 mouse mAb	MBL International (Watertown, MA)	human	IgG ₁
clone 3G4B2 mouse mAb	Upstate Biotechnology (Lake Placid, NY)	dog (MDCK cells)	IgG _{1k}

If additional anti-plasma membrane nucleolin antibodies are desired, they can be produced using well-known methods (Harlow and Lane, 1988; Harlow and Lane, 1999). For example, polyclonal antibodies (pAbs) can be raised in a mammalian host by one or more injections of an immunogen, such as an extracellular domain of surface-expressed nucleolin, and, if desired, an adjuvant. Typically, the immunogen (and adjuvant) is injected in a mammal by a subcutaneous or intraperitoneal injection. The immunogen may include components such as polypeptides (isolated, non-

isolated, or recombinantly produced), cells or cell fractions. Examples of adjuvants include Freund's complete, monophosphoryl Lipid A synthetic-trehalose dicorynomycolate, aluminum hydroxide (alum), heat shock proteins HSP 70 or HSP96 (WO01/917871A1), squalene emulsion containing monophosphoryl lipid A (LaPosta and Eldridge, 2001), α_2 -macroglobulin and surface active substances, including oil emulsions, pleuronic polyols, polyanions and dinitrophenol. To improve the immune response, an immunogen may be conjugated to a polypeptide that is immunogenic in the host, such as keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, cholera toxin, labile enterotoxin, or soybean trypsin inhibitor. Alternatively, pAbs may be made in chickens, producing IgY molecules (Schade *et al.*, 1996).

Monoclonal antibodies (mAbs) may also be made by immunizing a host or lymphocytes from a host, harvesting the mAb-secreting (or potentially secreting) lymphocytes, fusing those lymphocytes to immortalized cells (e.g., myeloma cells), and selecting those cells that secrete the desired mAb (Goding, 1996; Kohler and Milstein, 1975). Other techniques may be used, such as EBV-hybridoma technique (Cole *et al.*, 1985; Coligan, 1996). Techniques for the generation of chimeric antibodies by splicing genes encoding the variable domains of non-human antibodies to genes of the constant domains of human immunoglobulin result in "chimeric antibodies" that are substantially human at the amino acid level (Neuberger, Williams *et al.* 1984; Morrison, Johnson *et al.* 1984). If desired, the mAbs may be purified from the culture medium or ascites fluid by conventional procedures, such as protein A-sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, ammonium sulfate precipitation or affinity chromatography (Harlow and Lane, 1988; Harlow and Lane, 1999). Additionally, human monoclonal antibodies can be generated by immunization of transgenic mice containing a third copy IgG human trans-loci and silenced endogenous mouse Ig loci (Surani *et al.*, 1996) or using human-transgenic mice (Jakobovits *et al.*, 1998; Lonberg and Kay, 1998). Production of humanized monoclonal antibodies and fragments thereof can also be generated through phage display technologies (Winter, Griffiths *et al.* 1994).

An example of the production of a murine monoclonal antibody to human nucleolin according to described techniques (Kohler and Milstein, 1975) is as follows.

Female BALB/c mice (20-25 g) are injected intraperitoneally with 100 µg of antigen containing human nucleolin polypeptide, or portion thereof. Alternatively, the antigen includes murine cells which have been transformed to express human nucleolin. After 2 weeks, a second injection having 50 µg of antigen is injected. To test for production of anti-nucleolin antibodies, sera from the mice are used in immunohistologic screening. Mice displaying high blood serum levels of anti-nucleolin antibody receive a third injection (20 µg) of antigen. Four days later, the mice are sacrificed, their spleen cells isolated and fused with a myeloma line, e.g., P3X63Ag8.653 (American Type Tissue Collection; Manassas, VA). The resulting hybridoma cells are cultured, sub-cloned and selected for expression of antibodies having high affinities for nucleolin.

Non-immunogenic human-like or humanized polyclonal antibodies that bind to nucleolin may also be produced. These polyclonal antibodies can be made, for example, using phage display methods (Sharon, 1995), or by immunizing transgenic or genetically engineered animals capable of producing human polyclonal antibodies (Singh and Dias, 2002).

Antibodies suitable for therapy

Antibodies most ideal for use as therapeutics are those that are non-immunogenic when administered to subjects. Such antibodies have the advantages of exerting minimal side-effects, having long serum and biologic half-life, having wide bio-distribution, having high target specificity and high activity in engaging the effector phase of the immune system. These antibodies, when intended for human subjects, are commonly referred to as "humanized," "human," "chimeric," or "primatized" antibodies; these are comprised substantially (>70%) of human amino acid sequences.

Detection

An approach using antibodies to detect the presence of an antigen will include one or more, if not all, of the following steps:

- (1) Preparing the entity being tested for plasma membrane nucleolin by washing with buffer or water

(2) Blocking non-specific antibody binding sites
(3) Applying the antibody (e.g., nucleolin)
(4) Detecting bound antibody, either *via* a detectable labeled-secondary antibody that recognizes the primary antibody or a detectable label that has been directly attached to, or associated with, the bound (anti-nucleolin) antibody.

5 Substrates may be washed with any solution that does not interfere with the epitope structure. Common buffers include saline and biological buffers, such as bicine, tricine, and Tris.

10 Non-specific binding sites are blocked by applying a protein solution, such as bovine serum albumin (BSA; denatured or native), milk proteins, or in the cases wherein the detecting reagent is a secondary antibody, normal serum or immunoglobulins from a non-immunized host animal whose species is the same origin as the detecting antibody. For example, a procedure using a secondary antibody made in goats would employ normal goat serum (NGS).

15 The substrate is then reacted with the antibody of interest. The antibody may be applied in any form, such as F_{ab} fragments and derivatives thereof, purified antibody (by affinity, precipitation, *etc.*), supernatant from hybridoma cultures, ascites, serum or recombinant antibodies expressed in recombinant cells. The antibody may be diluted in buffer or media, often with a protein carrier such as the solution used to block non-specific binding sites; the useful antibody concentration is usually determined empirically. In general, polyclonal sera, purified antibodies and ascites may be diluted 1:50 to 1:200,000, more often, 1:200 to 1:500. Hybridoma supernatants may be diluted 1:0 to 1:10, or may be concentrated by dialysis or ammonium sulfate precipitation (or any other method that retains the antibodies of interest but at least partially removes the liquid component and preferably other small molecules, such as salts) and diluted if necessary. Incubation with antibodies may be carried out for as little as 20 minutes at 37°C, 2 to 6 hours at room temperature (approximately 22°C), or 8 hours or more at 4°C.

20 25

25 To detect an antibody-antigen complex, a label may be used. The label may be coupled to the binding antibody, or to a second antibody that recognizes the first antibody, and is incubated with the sample after the primary antibody incubation and thorough washing. Suitable labels include fluorescent moieties, such as fluorescein

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5 isothiocyanate; fluorescein dichlorotriazine and fluorinated analogs of fluorescein; naphthofluorescein carboxylic acid and its succinimidyl ester; carboxyrhodamine 6G; pyridyloxazole derivatives; Cy2, 3 and 5; phycoerythrin; fluorescent species of succinimidyl esters, carboxylic acids, isothiocyanates, sulfonyl chlorides, and dansyl chlorides, including propionic acid succinimidyl esters, and pentanoic acid succinimidyl esters; succinimidyl esters of carboxytetramethylrhodamine; rhodamine Red-X succinimidyl ester; Texas Red sulfonyl chloride; Texas Red-X succinimidyl ester; Texas Red-X sodium tetrafluorophenol ester; Red-X; Texas Red dyes; tetramethylrhodamine; lissamine rhodamine B; tetramethylrhodamine; tetramethylrhodamine isothiocyanate; naphthofluoresceins; coumarin derivatives; pyrenes; pyridyloxazole derivatives; dapoxyl dyes; Cascade Blue and Yellow dyes; benzofuran isothiocyanates; sodium tetrafluorophenols; 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene. Suitable labels further include enzymatic moieties, such as alkaline phosphatase or horseradish peroxidase; radioactive moieties, including ^{35}S and ^{135}I .
10 labels; avidin (or streptavidin)-biotin-based detection systems (often coupled with enzymatic or gold signal systems); and gold particles. In the case of enzymatic-based detection systems, the enzyme is reacted with an appropriate substrate, such as 3, 3'-diaminobenzidine (DAB) for horseradish peroxidase; preferably, the reaction products are insoluble. Gold-labeled samples, if not prepared for ultrastructural analyses, may be chemically reacted to enhance the gold signal; this approach is especially desirable for
15 light microscopy. The choice of the label depends on the application, the desired resolution and the desired observation methods. For fluorescent labels, the fluorophore is excited with the appropriate wavelength and the sample observed using a microscope, confocal microscope, or FACS machine. In the case of radioactive labeling, the samples are contacted with autoradiography film, and the film developed; alternatively,
20 autoradiography may also be accomplished using ultrastructural approaches. Alternatively, radioactivity may be quantified using a scintillation counter.
25 Also disclosed are methods for the detection of a tumor in an individual *in vivo*. The individual is administered a pharmaceutically acceptable composition having an agent that binds cell-surface nucleolin. The agent also incorporates a detectable label, such as a radiolabel, which distribution in the subject's
30

body is then mapped. The agent may comprise an aptamer, an oligonucleotide, a peptide, a small molecule, or a macromolecule, such as an antibody.

In one example of the method, a composition that specifically binds to tumor cell surface nucleolin is radiolabeled. The nature of the radiolabel is determined by the device that is used to record the presence and distribution of the agent in the patient's body. For example, if standard radio-imaging using gamma cameras is used, any one of several radioisotopes is used, such as technetium-99 or indium-111. Alternatively, if positron emission tomography (PET) is used, then the radiolabel is a radioactive halogen, such as flourine-18. The patient is injected intravenously with the radiolabeled agent, and scans of the patient, to visualize the location of the agent, are performed over the following 12 hour period, at, for example, 1, 4, and 12 hours post-injection. Monitoring techniques include whole body scanning, SPECT (which allows cross-sections of the body to be visualized), and any technique that permits monitoring of emission.

15

Cytological-based approaches:

Immunofluorescence/immunohistochemical

Protein expression by cells or tissue can be ascertained by immunolocalization of an antigen. Generally, cells or tissue are preserved by fixation, exposed to an antibody that recognizes the epitope of interest, such as a nucleolin, and the bound antibody visualized.

25

Any cell, cell line, tissue, or even an entire organism is appropriate for fixation. Cells may be cultured *in vitro* as primary cultures, cell lines, or harvested from tissue and separated mechanically or enzymatically. Tissue may be from any organ, plant or animal, and may be harvested after or prior to fixation. Fixation, if desired, may be by any known means; the requirements are that the protein to be detected be not rendered unrecognizable by the binding agent, most often an antibody. Appropriate fixatives include paraformaldehyde-lysine-periodate, formalin, paraformaldehyde, methanol, acetic acid-methanol, glutaraldehyde, acetone, Karnovsky's fixative, *etc.* The choice of fixative depends on variables such as the protein of interest, the properties of a particular detecting reagent (such as an antibody), the method of detection (fluorescence, enzymatic) and the method of

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observation (epifluorescence microscopy, confocal microscopy, light microscopy, electron microscopy, *etc.*). The sample is usually first washed, most often with a biological buffer, prior to fixation. Fixatives are prepared in solution or in biological buffers; many fixatives are prepared immediately prior to applying to the sample.

5 Suitable biological buffers include saline (e.g., phosphate buffered saline), N-(carbamoylmethyl)-2-aminoethanesulfonic acid (ACES), N-2-acetamido-2-iminodiacetic acid (ADA), bicine, bis-tris, 3-cyclohexylamino-2-hydroxy-1-propanesulfonic acid (CAPSO), ethanolamines, glycine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2-N-morpholinoethanesulfonic acid (MES), 3-N-morpholincpropanesulfonic acid (MOPS), 3-N-morpholino-2-hyrdoxypropanesulfonic acid (MOPSO), piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), tricine, triethanolamine, *etc.* An appropriate buffer is selected according to the sample being analyzed, appropriate pH, and the requirements of the detection method. A useful buffer is phosphate buffered saline (PBS). After fixation, the sample may be stored in fixative, preferably fresh, or temporarily or indefinitely, at a temperature between about 4° C to about 22° C.

10 After fixation from 5 minutes to 1 week, depending on the sample size, sample thickness, and viscosity of the fixative, the sample is washed in buffer. If the sample is thick or sections are desired, the sample may be embedded in a suitable matrix. For cryosectioning, sucrose is infused, and embedded in a matrix, such as OCT Tissue 15 Tek (Andwin Scientific; Canoga Park, CA) or gelatin. Samples may also be embedded in paraffin wax, or resins suitable for electron microscopy, such as epoxy-based (Araldite, Polybed 812, Durcupan ACM, Quctol, Spurr's, or mixtures thereof; Polysciences, Warrington, PA), acrylates (London Resins (LR White, LR gold), 20 Lowicryls, Unicryl; Polysciences), methylacrylates (JB-4, OsteoBed; Polysciences), melamine (Nanoplast; Polysciences) and other media, such as DGD, Immuno-Bed 25 (Polysciences) and then polymerized. Resins that are especially appropriate include hydrophilic (such as Lowicryls, London Resins, water-soluble Durcupan, *etc.*) since these are less likely to denature the protein of interest during polymerization and will not repel antibody solutions. When embedded in wax or resin, samples are 30 dehydrated by passing them through a concentration series of ethanol or methanol; in some cases, other solvents may be used, such as polypropylene oxide. Embedding

may occur after the sample has been reacted with the detecting agents, or samples may be first embedded, sectioned (via microtome, cyrotome, or ultramicrotome), and then the sections reacted with the detecting reagents. In some cases, the embedding material may be partially or completely removed before detection to facilitate antigen access.

5 In some instances, the nucleolin epitope(s) to which the antibody binds may be rendered unavailable because of fixation. Antigen retrieval methods can be used to make the antigen available for antibody binding. Many recourses are available (reviewed in, for example, (McNicol and Richmond, 1998; Robinson and Vandre, 10 2001; Shi *et al.*, 2001)). Common methods include using heat supplied from autoclaves, microwaves, hot water or buffers, pressure cookers, or other sources of heat. Often the sources of heat are used in sequence; the samples must often be in solution (*e.g.*, microwave treatments). Detergent treatment may also unmask antigens, such as sodium dodecyl sulfate (SDS, 0.25% to 1%) or other denaturing detergents. Chemical methods include strong alkalis (such as NaOH), prolonged 15 immersion in water, urea, formic acid and refixation in zinc sulfate-formalin. In other instances, proteolytic enzyme treatment will modify the antigen such that it is available to the antibody. Any number of proteases may be used, such as trypsin. These methods may be combined to achieve optimal results. The choice of the 20 antigen retrieval method will depend on the sample, its embedment (if any), and the anti-nucleolin antibody.

Especially in the cases of immunofluorescent or enzymatic product-based detection, background signal due to residual fixative, protein cross-linking, protein precipitation or endogenous enzymes may be quenched, using, *e.g.*, ammonium 25 chloride or sodium borohydride or a substance to deactivate or deplete confounding endogenous enzymes, such as hydrogen peroxide which acts on peroxidases. To detect intracellular proteins in samples that are not to be sectioned, samples may be permeabilized. Permeabilizing agents include detergents, such as t-octylphenoxypolyethoxyethanols, polyoxyethylenesorbitans, and other agents, such as 30 lysins, proteases, *etc.*

Non-specific binding sites are blocked by applying a protein solution, such as bovine serum albumin (BSA; denatured or native), milk proteins, or preferably in the

cases wherein the detecting reagent is an antibody, normal serum or IgG from a non-immunized host animal whose species is the same is the same origin of the detecting antibody.

Flow cytometry/Fluorescence-Activated Cell Sorting (FACS)

5 Methods of performing flow cytometry are well known (Orfao and Ruiz-Arguelles, 1996). Because plasma membrane nucleolin is being probed, cell permeabilization that allows access to cytoplasmic compartments is undesirable. After harvesting, cells are prepared as a single-cell suspension; cells are then 10 incubated with an anti-nucleolin antibody usually after blocking non-specific binding sites. Preferably, the anti-nucleolin antibody is labeled with a fluorescent marker. If the antibody is not labeled with a fluorescent marker, a second antibody that is immunoreactive with the first antibody and contains a fluorescent marker is used. After sufficient washing to ensure that excess or unbound antibodies are removed, the cells are ready for flow cytometry.

15 Biochemical-based approaches:

In these approaches, it is first desirable to isolate plasma membrane proteins from other cellular compartments. This may be done in any number of ways, such as 20 simple cell extraction, differential extraction or mechanical disruption followed by separation of cellular compartments on gradients (such as sucrose or polydextran) by centrifugation, extraction followed by immunoselecting appropriate cellular compartments with plasma membrane-specific antibodies, *etc.* An example of such an approach is described in (Naito *et al.*, 1988; Yao *et al.*, 1996b)). Extracting reagents are well known. For examples, solvents such as methanol may be 25 occasionally useful. More likely, detergents, such as t-octylphenoxypolyethoxyethanol (also known as polyethylene glycol tert-octylphenyl ether) are particularly useful for simple extractions. Also useful are glucopyranosides, maltopyranosides, maltosides, polyoxyethylene esters, other polyoxyethylene ethers, salts of alginic, caprylic, cholic 1-decanesulfonic, 30 deoxycholic, dioctyl sulfosuccinate, 1-dodecanesulfonic, glyocholic, glycodeoxycholic, 1-heptanesulfonic, 1-hexanesulfonic, N-lauroylsacrosine, lauryl sulfate (e.g., SDS), 1-nananesulfonic, 1-octanesulfonic, 1-pentanesulfonic,

taurocholic and tauodexychoic acids; sodium 7-ethyl-2-methyl-4-undecyl sulfate, and sodium 2-ethylhexyl sulfate. Other useful detergents include(3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane-sulfonate, N-decyl-, N-dodecyl-, N-hexadecyl-, N-octadecyl-, N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonates and phosphatidylcholine. Less useful, but may be helpful in some cases, are alkyltrimethylammonium bromides, benzalkonium chloride, benzethonium chloride, benzylidemethyldodecylammonium bromide, benzylidemethylhexadecylammonium chloride, cetyltrimethylammonium bromide, cetylpyridinium, decamethonium bromide, dimethylidioctadecylammonium bromide, methylbenzethonium chloride, methyltiroctylammonium chloride, and N,N',N'-polyoxyethylene(10)-N-tallow-1,3-diaminopropane. The different extracting reagents may be used singly or in combination; they may be prepared in simple aqueous solutions or suitable buffers.

15 Polymethylene glycol ter-octylphenyl ether is particularly useful for differential extraction by taking advantage of the low cloud point to separate membrane proteins from soluble proteins into two different phases.

Extraction buffers may contain protease inhibitors, such as aprotinin, benzamidine, antipain, pepstatin and iodoacetamide.

20 Extracts are then assayed for nucleolin expression. For those techniques that separate surface plasma membrane from other cellular components (especially the nucleus), the nucleolin detecting agents need not be specific for extracellular plasma membrane nucleolin epitopes.

Immunosorbent assay (ELISA) (Ausubel *et al.*, 1987)

25 Various types of enzyme linked immunosorbent assays (ELISAs) to detect protein expression are known, and these are applicable to nucleolin detection. However, other ELISA-like assays include radio-immunoassays and other non-enzyme linked antibody binding assays and procedures. In these assays, the cell surface proteins are the principle components in the cell preparation.

30 The double antibody-sandwich ELISA technique is especially useful. The basic protocol for a double antibody-sandwich ELISA is as follows: A plate is coated with anti-nucleolin antibodies (capture antibodies). The plate is then washed with a

5 blocking agent, such as BSA, to block non-specific binding of proteins (antibodies or antigens) to the test plate. The test sample is then incubated on the plate coated with the capture antibodies. The plate is then washed, incubated with anti-nucleolin antibodies, washed again, and incubated with a specific antibody-labeled conjugates and the signal appropriately detected.

10 In other ELISAs, proteins or peptides are immobilized onto a selected surface, the surface exhibit may have affinity for proteins, such as the wells of a specially-treated polystyrene microtiter plate. After washing to remove incompletely adsorbed material, one would then generally desire to bind or coat with a nonspecific protein that is known to be antigenically neutral with anti-nucleolin antibodies, such as BSA 15 or casein, onto the well bottom. This step allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antibodies onto the surface. When the antibodies were created in an animal by conjugating a polypeptide to a protein (e.g., BSA), a different protein is usually used as a blocking agent, because of the possibility of the presence of antibodies to the blocking protein the antibody composition.

20 After binding of nucleolin to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with an anti-nucleolin antibody composition in a manner conducive to immune complex (antigen/antibody) formation. Such conditions include 25 diluting the antibody composition with diluents such as BSA, bovine γ globulin (BGG) and PBS/Polyoxyethylenesorbitan monolaurate. These added agents also assist in the reduction of nonspecific background signal. The layered antibody composition is then allowed to incubate for, e.g., from 2 to 4 hours at 25° C to 37° C. Following incubation, the antibody composition-contacted surface is washed so as to remove non-immunocomplexed material. One washing procedure includes washing 30 with a PBS/polyoxyethylenesorbitan monolaurate or borate buffer solution.

Following formation of specific immunocomplexes between the test sample and the antibody and subsequent washing, immunocomplex formation is detected using a second antibody having specificity for the anti-nucleolin antibody. For detection, the secondary antibody is associated with detectable label, such as an

enzyme or a fluorescent molecule. A number of immunoassays are discussed in U.S. Patent Nos. 5,736,348, 5,192,660, and 4,474,892.

Western blotting (Ausubel et al., 1987)

5 Western blotting methods are well known. Generally, a protein sample is subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at such conditions as to yield an appropriate separation of proteins within the sample. The proteins are then transferred to a membrane (e.g., nitrocellulose, nylon, etc.) in such a way as to maintain the relative positions of the proteins to each other.

10 Visibly labeled proteins of known molecular weight may be included within a lane of the gel. These proteins serve as a control to insure adequate transfer of the proteins to the membrane, as well as molecular weight markers for determining the relative molecular weight of other proteins on the blot. Alternatively, unlabeled marker proteins (or in some rare instances, no marker proteins) are detected after transfer with Brilliant Blue (G or R; Sigma; St. Louis, MO) other protein dyes. After 15 protein transfer, the membrane is submersed in a blocking solution to prevent nonspecific binding of the primary antibody.

20 The primary antibody, e.g., anti-nucleolin, may be labeled and the presence and molecular weight of the antigen may be determined by detecting the label at a specific location on the membrane. However, the primary antibody may not be labeled, and the blot is further reacted with a labeled second antibody. This secondary antibody is immunoreactive with the primary antibody; for example, the secondary antibody may be one to rabbit immunoglobulins and labeled with alkaline phosphatase. An apparatus for and methods of performing Western blots are described in U.S. Patent No. 5,567,595.

25 Immunoprecipitation (Ausubel et al., 1987; Harlow and Lane, 1999)

30 Protein expression can be determined and quantified by isolating antigens using immunoprecipitation. Methods of immunoprecipitations are described in U.S. Patent No. 5,629,197. Immunoprecipitation involves the separation of the target antigen component from a complex mixture and is used to discriminate or isolate minute amounts of protein. For the isolation of cell-surface proteins, nonionic salts are often used.

For example, an immunoprecipitation from whole cells may be performed as follows. Cells are extracted with one or more detergents (see above), such as, for example, 1% t-octylphenoxypolyethoxyethanol/0.1% SDS/150 mM NaCl in 20 mM Tris buffer, pH 8.6. After extraction, which may be aided by agitation, insoluble debris is removed using a centrifuge. Anti-nucleolin antibody is added to the extracts, and then the samples are incubated 30 minutes to overnight at 4° C. *Staphylococcus aureus* or recombinantly-produced Protein A or Group C *Staphylococcus* Protein G conjugated to sepharose or tris-acryl beads are then added. In those instances when the anti-nucleolin antibody does not bind well to Protein A, IgG Abs that recognize antibodies of the animal in which the anti-nucleolin antibody was made is simultaneously added. The samples are then incubated with gentle agitation for around 2 hours at 4° C. The beads or bacterial cells, now bound to the antibody-antigen complexes, are thoroughly washed, usually first with either the extraction solution or a high salt buffer, then a salt-less buffer or water to remove nonspecifically-bound proteins and residual detergent molecules. After removing residual buffer, the beads are incubated with a buffer, such as electrophoresis sample buffer, and then subjected to 95°C for 3-5 minutes to elute bound proteins from the beads. The samples are then ready for analysis and nucleolin detection.

Other methods:

Immunoselection procedures (other than FACS) (Ausubel *et al.*, 1987)

Cells expressing plasma membrane nucleolin can be easily isolated by "panning" on plastic plates coated with anti-antibody antibodies (Wysocki and Sato, 1978). Panning has many advantages over other immunoselection procedures: It is fast, efficient (10⁷ cells can easily be panned on two 60-mm plastic plates in 30 minutes), and inexpensive.

In general, a single cell suspension is labeled with an anti-nucleolin antibody, and then is incubated on a substrate coated with a secondary antibody (with non-specific binding sites blocked). After 1 to 3 hours incubation at room temperature, non-adherent cells are washed away. In this embodiment, bound cells indicate that nucleolin is expressed in the plasma membrane, indicating a neoplastic cell.

Detecting nucleolin: Oligonucleotide-based methods

5 GROs and other oligonucleotides that recognize and bind nucleolin (Bates *et al.*, 1999; Miller *et al.*, 2000; Xu *et al.*, 2001) can be used much the same way as antibodies are. Examples of suitable assays are given below. In some cases, incorporating the GRO nucleotides into larger nucleic acid sequences may be advantageous; for example, to facilitate binding of a GRO nucleic acid to a substrate without denaturing the nucleolin-binding site.

10 Useful GROs that bind nucleolin (and also have the biological property of inhibiting cancer cell growth) have been described (Bates *et al.*, 1999; Miller *et al.*, 2000; Xu *et al.*, 2001). They include those shown in Table 2. Control GROs are useful for detecting background signal levels.

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Table 2 Non-anti-sense GRO that bind nucleolin and non-binding controls^{1,2,3}

८८

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SEQ ID NO:	Sequence	GRN
18	tttcctcctc ctcatttcotcc tccttcctcc	CRO
19	tttagatgttg ggtttaggtt aagg	GRO A
20	ggtaggttgtg g	GRO B
21	ggtaggttgtg tttag	GRO C
22	ggtaggttgtg gtgtg	GRO D
23	gggtttttggg	GRO E
24	ggtttttgtt ttgggtttgg	GRO F
25	ggttgggttg gtgtg	GRO G
26	ggggttttgg gg	GRO H
27	gggtttttgg	GRO I
28	ggggttttgg ggtttttggg ttttttgg	GRO J
29	ttgggtttgg gttttttgggtt ggtgg	GRO K
30	gggggggg ggtaggt	GRO L
31	ggtttttgtt ttgggtttgg ttttttgg	GRO M
32	tttcctcctc ctcatttcotcc tccttcctcc	GRO N ²
33	ccttcctcctc ctttttcotcc tccttcctcc	GRO O ²
34	ttgggt	GRO P ²
35	ggatgtct	GRO Q ²

34

"Express Mail" mailing label number EV 52528 1827 US Date of Deposit: October 8, 2004

GRO	Sequence	SEQ ID NO:
GRO R ²	gccgtttgcg g	36
GRO S ²	ttag	37
GRO T ²	gggtttgggg ttttttttgg 939	38
¹ Indicates a good plasma membrane nucleolin-binding GRO. ² Indicates a nucleolin control (non-plasma membrane nucleolin binding). ³ GRO sequence without ¹ or ² designations have some anti-proliferative activity.		

Cytological-based approaches:

Cellular localization/labeling (relative of immuno-based localization/labeling assays)

The procedures outlined above for the immuno-based localization assays (such as immunofluorescence or FACS) are also applicable to those assays wherein the detecting reagent is a nucleolin-binding GRO. Modifications include those to prevent non-specific binding, using denatured DNA, such as from salmon sperm instead of a protein such as BSA. For detection, similar labels as outlined above are also useful as long as the GRO can be derivatized with the label in some form. For this purpose, biotin-avidin nucleic acid labeling systems are especially convenient, as are

digoxigenin ones (Ausubel *et al.*, 1987). The synthesis of biotinylated nucleotides has been described (Langer *et al.*, 1981). Biotin, a water-soluble vitamin, can covalently attach to the C5 position of the pyrimidine ring via an alylamine linker arm; biotin non-covalently binds avidin or streptavidin, which may be easily labeled.

Alternatively, biotin is added to oligonucleotides during synthesis by coupling to the 5'-hydroxyl of the terminal nucleotide. Digoxigenin-11-dUTP can be incorporated into DNA by either nick translation or random oligonucleotide-primed synthesis protocols. Digoxigenin is detected using labeled anti-digoxigenin antibodies.

Convenient digoxigenin systems are commercially available (Roche Molecular Biochemicals; Indianapolis, IN). An example of a procedure using oligonucleotides to detect and localize proteins has been described by (Davis *et al.*, 1998).

Biochemical-based approaches:

GROs may also be used in a similar fashion as antibodies to detect nucleolin in biochemical approaches, as described above. For example, "Southwestern"-type blotting experiments may be performed with GROs (Bates *et al.*, 1999; Miller *et al.*, 2000). After cells have been appropriately extracted (for example, differentially to separate plasma membrane proteins from intracellular proteins), the proteins are subjected to electrophoresis on polyacrylamide gels and transferred to a substrate, such as a polyvinylidene difluoride membrane. Proteins are denatured and renatured by washing for 30 minutes at 4° C with 6 M guanidine-HCl, followed by washes in 3 M, 1.5 M and 0.75 M guanidine HCl in 25 mM HEPES (pH 7.9)/4 mM KCl/3 mM MgCl₂). After blocking non-specific binding sites with 5% non-fat dried milk in

HEPES buffer, the labeled GRO is hybridized for 2 hours at 4°C in HEPES binding buffer supplemented with 0.25% NDM, 0.05% NP-40, 400 ng/ml salmon sperm DNA and 100 ng/ml of an unrelated mixed sequence oligonucleotide, such as *tcgagaaaaa*
5 *ctctctcttc* *cttccttc* *ctcca*; SEQ ID NO:17. After washing with HEPES binding buffer, the signal is detected appropriately.

Other methods:

Arrays

Arrays of immobilized nucleolin-binding reagents on chips

10 A chip is an array of regions containing immobilized molecules, separated by regions containing no molecules or immobilized molecules at a much lower density. For example, a protein chip may be prepared by applying nucleolin-binding antibodies; an "aptamer"-like chip may be prepared by applying nucleolin binding GROs. The remaining regions are left uncovered or are covered with inert molecules.

15 The arrays can be rinsed to remove all but the specifically immobilized polypeptides or nucleic acids. In addition, chips may also be prepared containing multiple nucleolin-binding antibodies (Table 1), nucleic acids (such as GROs; Table 2), or both, and may contain control antibodies and/or nucleic acids that are non-reactive with nucleolin. Such an array would allow for simultaneous test confirmation, 20 duplication and internal controls.

25 Proteins, such as anti-nucleolin antibodies, can be immobilized onto solid supports by simple chemical reactions, including the condensation of amines with carboxylic acids and the formation of disulfides. This covalent immobilization of proteins on inert substrates can prevent high background signals due to non-specific adsorption. Substrates derivatized with other molecules, such as biotin, are also useful when the protein to be immobilized is derivatized with avidin or streptavidin, or *vice-versa*. In some rare cases, especially when anti-nucleolin antibody-encoding nucleic acid sequences are available, fusion polypeptides comprising anti-nucleolin antibody may be advantageous for immobilization onto a substrate.

30 The surface may be any material to which a the nucleolin binding agent can be immobilized. For example, the surface may be metal, glass, ceramic, polymer, wood or biological tissue. The surface may include a substrate of a given material and a

layer or layers of another material on a portion or the entire surface of the substrate. The surfaces may be any of the common surfaces used for affinity chromatography, such as those used for immobilization of glutathione for the purification of GST fusion polypeptides. The surfaces for affinity chromatography include, for example, sepharose, agarose, polyacrylamide, polystyrene and dextran. The surface need not be a solid, but may be a colloid, an exfoliated mineral clay, a lipid monolayer, a lipid bilayer, a gel, or a porous material.

5 The immobilization method desirably controls the position of the nucleolin binding agent on the surface; for example, enabling the antigen binding portions of antibodies unattached to the substrate, while the non-antigen binding portions are rooted to the substrate. By controlling the position of individual reactant ligands, patterns or arrays of the ligands may be produced. The portions of the surface that are not occupied by the nucleolin-binding reagent do not allow non-specific adsorption of polypeptides or polynucleotides.

10 15 In this embodiment, a sample from a subject, for example, blood, is passed over a chip containing nucleolin-binding molecules. A biosensing device, such as machine that detects changes in surface plasmon resonance, is then used to detect bound nucleolin. BIACore (Uppsala, Sweden) chips serve as examples of useful chips and detection machines.

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Prognostic assays

Diagnostic methods can furthermore be used to identify subjects having, or at risk of developing, a neoplasia at an early stage of disease development, since the surface expression of nucleolin can be detected earlier than in conventional methods.

30 25 Prognostic assays can be used to identify a subject having or at risk for developing a neoplasia, such as a subject who has a family history of harmful neoplasias, especially cancers. A method for identifying such an individual would include a test sample obtained from a subject and testing for cell surface localization of nucleolin.

In another embodiment, detecting plasma membrane nucleolin and then either qualitatively or quantitatively assessing the amount of nucleolin (usually indirectly through the signal generated from bound nucleolin molecules) can indicate the rate of

cell proliferation, since plasma membrane nucleolin levels correlate with cell proliferation rates.

Kits

5 Kits, containers, packs, or dispensers containing nucleolin probes and detection reagents, together with instructions for administration, may be assembled. When supplied as a kit, the different components may be packaged in separate containers and admixed immediately before use. Such packaging of the components separately may permit long-term storage without losing the active components' functions.

10 Kits may also include reagents in separate containers that facilitate the execution of a specific test, such as diagnostic tests. For example, non-nucleolin-binding GROs may be supplied for internal negative controls, or nucleolin and a nucleolin-binding reagent for internal positive controls. The components of a kit are an anti-nucleolin agent used to probe for nucleolin, a control sample, and optionally a composition to detect nucleolin. Examples of anti-nucleolin agents include an anti-nucleolin antibody (e.g., as shown in Table 1) or fragment thereof; if labeled, then a nucleolin-binding detection reagent is superfluous. A nucleolin-binding oligonucleotide (e.g., as shown in Table 2), which may be derivatized such that a second labeled reagent may bind (such as biotin). However, if a labeled GRO nucleic acid is provided, then a second labeled reagent is superfluous. Examples of detection reagents include: labeled secondary antibodies, for example, an anti-mouse pAb made in donkey and then tagged with a fluorophore such as rhodamine, or a labeled reagent to detect oligonucleotides such as GROs; for example, avidin or streptavidin linked to horseradish peroxidase when the probe is biotinylated. Control components may include: normal serum from the animal in which a secondary antibody was made; a solution containing nucleolin polypeptide or nucleolin binding oligonucleotide; a dot blot of nucleolin protein to assay nucleolin-binding reagent reactivity; or fixed or preserved cells that express nucleolin in the plasma membrane.

15 20 25 Other components may include buffers, fixatives, blocking solutions, microscope slides and/or cover slips or other suitable substrates for analysis, such as microtiter plates; detergent or detergent solutions or other cell extraction reagents; miscellaneous

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reagents, protease inhibitors, various containers and miscellaneous tools and equipment to facilitate the assays.

(a) *Containers or vessels*

The reagents included in the kits can be supplied in containers of any sort such that the life of the different components are preserved and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain lyophilized nucleolin binding reagents (such as anti-nucleolin antibodies or nucleolin-binding oligonucleotides) or buffers that have been packaged under a neutral, non-reacting gas, such as nitrogen. Ampules may consist of any suitable material, such as glass, organic polymers (*i.e.*, polycarbonate, polystyrene, *etc.*), ceramic, metal or any other material typically employed to hold reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes that may have foil-lined interiors, such as aluminum or alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like.

Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to mix. Removable membranes may be glass, plastic, rubber, *etc.*

(b) *Instructional materials*

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, DVD, videotape, audio tape, *etc.* Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

Methods of treatment

Therapeutic methods

Also disclosed are methods of modulating nucleolin expression or activity for therapeutic purposes. The modulatory method of the disclosure involves contacting a cell with an agent that modulates one or more of the

activities of nucleolin activity associated with the cell. An agent that modulates nucleolin activity can be a nucleic acid or a protein, a naturally occurring cognate ligand of nucleolin, a peptide, a nucleolin peptidomimetic, or other small molecule. 5 Modulatory methods can be performed *in vivo* (e.g., by administering the agent to a subject). As such, the invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant expression or activity of a nucleolin.

Anti-nucleolin antibodies as therapeutic agents
10 Any antibody, as described in "*Detecting nucleolin: antibody-based methods: Antibodies*," above, that binds and interferes with nucleolin may be used to treat tumors and cancers. In certain instances, monoclonal antibodies are preferred as they bind single, specific and defined epitopes. In other instances, however, polyclonal antibodies capable of interacting with more than one epitope on nucleolin are 15 preferred. The antibodies may be whole antibodies and fragments or derivatives thereof. For example, when assaying live cells, using F_{ab} fragments will eliminate cross-linking, thus preventing cells from endocytosing bound antibodies.

Spliceosome-mediated RNA trans-splicing (SMaRT) (Mitchell, 1997)
20 In another embodiment, a subset of cells expressing select members of nucleolin is targeted through spliceosome-mediated RNA trans-splicing. This method is a means for expressing a heterologous gene in a selected subset of cells by targeting a trans-splicing reaction between a precursor therapeutic molecule (PMT) and pre-mRNA molecules which are uniquely expressed in the specific target cells (Puttaraju, 25 DiPasquale *et al.* 2001). The heterologous gene can either be of therapeutic value to the cell or a toxin which kills the specific cells.

Anti-sense Compounds
30 Methods of treating a tumor in a subject include administering a therapeutically effective amount of an anti-sense nucleic acid molecule or ribozymes that may be used to modulate, particularly inhibit, the expression of nucleolin.

Anti-sense nucleic acid molecules are sequence-specific tools capable of selectively modifying or silencing gene expression. Anti-sense oligos function by binding complementary sequences of a specific gene's cognate RNA by Watson-Crick base pairing to form RNA-oligo hybrid molecules (Knorre and Vlassov 1990).
5 Formation of RNA-oligo hybrids interferes with RNA function, stability and consequently protein expression. Various mechanisms have been attributed to the inhibition of protein translation by anti-sense nucleic acid molecules including:
interference by physical steric effects and initiation of RNase H-mediated degradation
of the double stranded anti-sense-oligo-probe:mRNA hybrid (Dagle and Weeks
10 2000). Anti-sense oligonucleotide molecules thus are useful therapeutically and as a tool to validate drug targets.

A preferred embodiment of a nucleolin anti-sense DNA has at least 10 nucleotides, preferably between 15 to 25 nucleotides, or a length that binds complementary strands and are most easily formulated and delivered to target organs and cells. Synthetic anti-sense nucleotides preferably contain phosphoester analogs, such as phosphorothioate or thioesters rather than entirely natural phosphodiester bonds as these naturally occurring bonds are labile to nucleases (Shaw, Kent *et al.* 1991). The phosphorothioate class of oligonucleotides have the additional advantages of high solubility, ease of synthesis, maintenance of Watson-Crick nucleotide hydrogen bonding patterns and the ability to activate RNase H-mediated degradation of cellular mRNA (Stein, Tonkinson *et al.* 1991; Crooke 1993; Srinivasan and Iversen 1995; Bock, Griffin *et al.* 1992).

25 Ribozymes are enzymatic "catalytic" RNA molecules that are self-cleaving and self-splicing (Cech 1986; Altman 1990; Symons 1992). By combining catalytic domains of naturally occurring ribozymes with oligonucleotides specific for a target RNA molecule, artificial catalytic RNA molecules that cleave specific RNA targets can be made. A ribozyme contains at least two functional domains: (1) a specialized sequence for RNA specific binding; and, (2) a catalytic sequence responsible for RNA cleavage (Cech *et al.*, 1992).

30

Interfering RNA

5 Tumors and cancers may also be treated by interfering with expression of key regulatory genes by administering interfering RNA compositions. Several embodiments of this technology have now been described, such as synthetic interfering RNA duplexes, synthetic short hairpin RNA duplexes, and gene expression systems enabling the *in vivo* production and delivery of the interfering RNA molecule (Sharp and Zamore 2000; Bernstein, Caudy *et al.* 2001; Ketting, Fischer *et al.* 2001; Sharp 2001; McManus, Petersen *et al.* 2002; McManus and Sharp 2002; Paddison, Caudy *et al.* 2002; Paddison, Caudy *et al.* 2002) (Beach *et al.*, 2001; Fire *et al.*, 2003; Tuschl *et al.*, 2002; Tuschl *et al.*, 2001).

10

Combination therapies

In practising the above-described methods, the specific inhibitors (e.g., antibodies, anti-sense, ribozymes, PMTs or interfering RNAs, directed against nucleolin) can be used alone or, preferably, in combination with one another, or with other anti-tumor agents such as radiation, chemotherapeutics, and cytotoxic drugs. Such combination therapy achieves superior and synergistic therapeutic results.

Administration

Pharmaceutical compositions

20

A "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration (Remington 2000). Preferred examples of such carriers or diluents include water, saline, Ringer's solutions and dextrose solution. Supplementary active compounds can also be incorporated into the compositions.

25

General considerations

30

A pharmaceutical composition is formulated to be compatible with its intended route of administration, including intravenous, intradermal, subcutaneous, oral, inhalation, transdermal, transmucosal, and rectal administration. Solutions and suspensions used for parenteral, intradermal or subcutaneous application can include a

sterile diluent, such as water for injection, saline solution, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; buffers such as acetates, citrates or phosphates, and agents for the 5 adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

10 *Injectable formulations*
Pharmaceutical compositions suitable for injection include sterile aqueous solutions or dispersions for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, CREMOPHOR EL® (BASF; Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid so as to be administered using a syringe. Such compositions should be stable during manufacture and storage and must be preserved against contamination from microorganisms such as bacteria and fungi. The carrier can be a dispersion medium containing, for example, water, polyol (such as glycerol, propylene glycol, and liquid polyethylene glycol), and other compatible, suitable mixtures. Various antibacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, and thimerosal, can contain microorganism contamination. Isotonic agents such as sugars, polyalcohols, such as manitol, sorbitol, and sodium chloride can be included in the composition. Compositions that can delay 15 absorption include agents such as aluminum monostearate and gelatin.
20 Sterile injectable solutions can be prepared by incorporating the anti-nucleolin agents, and other therapeutic components, in the required amount in an appropriate solvent with one or a combination of ingredients as required, followed by sterilization. Methods of preparation of sterile solids for the preparation of sterile injectable 25 compositions include vacuum drying and freeze-drying to yield a solid.
30

Oral compositions

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included. Tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, PRIMOGEL®, or corn starch; a lubricant such as magnesium stearate or STEROTES®; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

15 *Compositions for inhalation*

For administration by inhalation, the compounds are delivered as an aerosol spray from a nebulizer or a pressurized container that contains a suitable propellant, e.g., a gas such as carbon dioxide.

20 *Carriers*

In one embodiment, the active compounds are prepared with carriers that protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable or biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such materials can be obtained commercially from ALZA Corporation (Mountain View, CA) and NOVA Pharmaceuticals, Inc. (Lake Elsinore, CA), or prepared by one of skill in the art.

30 *Transmucosal or transdermal formulations*

Administration can be transmucosal or transdermal. For transmucosal or transdermal administration, penetrants that can permeate the target barrier(s) are

selected. Transmucosal penetrants include, detergents, bile salts, and fusidic acid derivatives. Nasal sprays or suppositories can be used for transmucosal administration. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams. Suppositories (e.g., with bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery may also be prepared.

Unit dosage
Oral formulations or parenteral compositions in unit dosage form can be created to facilitate administration and dosage uniformity. Unit dosage form refers to physically discrete units suited as single dosages for the subject to be treated, containing a therapeutically effective quantity of active compound in association with the required pharmaceutical carrier. The specification for the unit dosage forms of the invention are dictated by, and directly dependent on, the unique characteristics of the active compound and the particular desired therapeutic effect, and the inherent limitations of compounding the active compound.

Dosage
The pharmaceutical composition may further comprise other therapeutically active compounds as noted herein which are usually applied in the treatment of cancers and tumors.
In the treatment or prevention of conditions which require nucleolin modulation, an appropriate dosage level of the therapeutic agent will generally be about 0.01 to 500 mg per kg patient body weight per day which can be administered in single or multiple doses. Preferably, the dosage level will be about 0.1 to about 250 mg/kg per day; more preferably about 0.5 to about 100 mg/kg per day. A suitable dosage level may be about 0.01 to 250 mg/kg per day, about 0.05 to 100 mg/kg per day, or about 0.1 to 50 mg/kg per day. Within this range the dosage may be 0.05 to 0.5, 0.5 to 5 or 5 to 50 mg/kg per day. For oral administration, the compositions are preferably provided in the form of tablets containing 1.0 to 1000 milligrams of the active ingredient, particularly 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, 75.0, 100.0, 150.0, 200.0, 250.0, 300.0, 400.0, 500.0, 600.0, 750.0, 800.0, 900.0, and 1000.0 milligrams

of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. The compounds may be administered on a regimen of 1 to 4 times per day, preferably once or twice per day.

5 However, the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

10

Determination of the biological effect of the therapeutic

Suitable *in vitro* or *in vivo* assays can be performed to determine the effect of a specific therapeutic and whether its administration is indicated for treatment of the affected tissue.

15

In various specific embodiments, *in vitro* assays may be performed with representative cells of the type(s) involved in the patient's disorder, to determine if a given therapeutic exerts the desired effect upon the cell type(s). Modalities for use in therapy may be tested in suitable animal model systems including, but not limited to rats, mice, chicken, cows, monkeys, rabbits, dogs and the like, prior to testing in human subjects. Similarly, for *in vivo* testing, any of the animal model system known in the art may be used prior to administration to human subjects.

20

The following examples are intended to illustrate the present invention without limitation.

25

EXAMPLES

Example 1 Immunofluorescent labeling of plasma membrane nucleolin in cells

This example illustrates a procedure that stains nuclear nucleolin, or only plasma membrane nucleolin.

30

Cells from the cell lines DU145 (human prostate cancer), MDA-MB-231 (human breast cancer) HeLa (human cervical cancer) and HS27 (normal skin fibroblasts) (all available from ATCC; Manassas, VA) were released from culture

substrates with trypsin, resuspended into single cells and plated onto microscope slides. The slides seeded with cells were incubated at 37°C until they were well attached, as assayed by visual inspection using a microscope. After rinsing the attached cells once with PBS for two minutes, they were fixed in 4% formaldehyde/PBS for at least 15 minutes at 22°C. For nuclear nucleolin staining, cells are permeabilized with 1% Triton X-100 prior to contacting with antibody. After washing twice with PBS, 5 minutes/wash, non-specific binding sites were blocked for 15-60 minutes with 1% NGS/PBS at 22°C, and then incubated with mouse anti-nucleolin antibodies diluted in 1% NGS/PBS or PBS/Tween (0.05%-0.1%) for 1 hour to overnight at 4°C. The samples were washed four times, 5 minutes each with PBS, and then incubated with goat anti-mouse pAb labeled with FITC-labeled secondary antibodies diluted in PBS for 1 hour at 22°C. After again washing four times with PBS for 5 minutes each, the samples were mounted in Mowiol mounting media (prepared as follows: 9 ml/glycerol and 3.36 g Mowiol 40-88 were agitated for 1 h at 22°C. Then, 9 ml of water was then added, and agitation continued for 2 h at 22°C. Tris (0.2 M, pH 8.5; 18 ml) was then added, and the solution incubated for 6 h at 50°C until the solids were almost completely dissolved. After centrifugation at 5,000 x g, the liquid phase was used for mounting), observed under a microscope, and photographed.

Figures 1 and 2 show nuclear (Figure 1) and plasma membrane (Figure 2) nucleolin staining in the various cell lines. Shown are immunofluorescent (Figures 1 and 2; panels B, D, F, H) and parallel phase contrast micrographs (Figures 1 and 2; panels A, C, E, G); DU145 cells are shown in A and B; MDA-MB-231 cells are shown in C and D; HeLa cells are shown in E and F; and HS27 cells are shown in G and H. All cell lines show clear nuclear nucleolin staining (Figures 1A, 1C, 1E and 1G). Note that when the cells are not permeabilized, thus restricting antibody access to the surface plasma membrane, the normal skin cell line, HS27, is completely negative for plasma membrane staining (Figure 2H) while cancer cells show strong staining (Figures 2B, 2D, 2F and 2H). Staining plasma membrane nucleolin is thus a superior method for diagnosis and prognosis compared to nuclear nucleolin or silver-staining NORs.

Example 2 Correlation of the degree of plasma membrane nucleolin expression and cancer aggressiveness

This experiment demonstrates that cell lines with high levels of plasma membrane nucleolin correspond to those with the most rapid proliferation.

5 Two cancer cell lines, DU145 and HeLa, and one normal cell line, HS27, were assayed for proliferation rate and compared. Cell doubling time is calculated by determining cell density at regular intervals using the MTT assay (based upon the ability of living cells to reduce 3-(van de Loosdrecht *et al.*, 1994)-2,5 diphenyltetrazolium bromide (MTT) into formazan; (van de Loosdrecht *et al.*, 1994)), and confirmed by counting the cells using trypan blue exclusion.

10 Figure 3 shows the comparative proliferation rates of DU145 (squares), HeLa (diamonds) and HS27 (circles) as measured by MTT assay. Until 3 days of culture, growth rates are similar, but after 3 days, HeLa and DU145 increase at a faster rate than the normal HS27 cells. Although MDA-MB-231 was not included in this 15 experiment, proliferation rate has been determined to be DU145 > MDA-MB-231 > HeLa > HS27. Note that the cell lines with high levels of plasma membrane nucleolin (see Figure 2) correspond to those with the most rapid proliferation (DU145 and HeLa).

20 *Example 3 Immunofluorescent labeling of nucleolin in paraffin-embedded tissue sections*

This example provides a suitable technique to detect and localize nucleolin in a fixed sample that has been embedded.

25 Sections of cells fixed and embedded in paraffin wax and anchored on microscope slides were washed in three changes of xylene (2 minutes each) to remove the paraffin, hydrated in graded alcohols (series 100%, 95% and 70%; 2 minutes each), and placed in PBS for 5 minutes. Antigen recovery used the approach of *low temperature antigen retrieval* (LTAR; (Shi *et al.*, 1997; Shi *et al.*, 2001)): After 30 digestion with 0.1% trypsin-EDTA (v/v) (Invitrogen Corp.; Carlsbad, CA) diluted in PBS for 15 minutes at 37° C/5% CO₂, the samples were washed with deionized water and incubated in 10 mM citrate buffer (pH 6) for 2 hours at 80° C. After cooling, the slides were rinsed with deionized water and then PBS.

5 Non-specific binding sites were blocked by incubation in 3% BSA in PBS for 30 minutes at 22° C. The samples were then incubated with 4 µg/ml mouse anti-nucleolin mAb (Santa Cruz) diluted in PBS/1% NGS at 4° C overnight. The samples were then brought to 22° C, washed four times with PBS for 5 minutes, and then reacted with 50 µg/ml Alexa488-conjugated goat anti-mouse IgG (Molecular Probes; Eugene, OR) and 2 µg/ml propidium iodide diluted in PBS/1% NGS for 1 hour at 22° C. After washing four times with PBS for 5 minutes, the samples were mounted in Mowiol mounting medium and observed under a fluorescent microscope.

10 Figure 4 shows the results of such an experiment. A clinical sample of a squamous cell carcinoma of the head and neck was prepared and probed for plasma membrane nucleolin. Plasma membrane nucleolin signal was relegated to malignant, neoplastic cells. Figure 4A shows the immunofluorescent signal obtained from probing for nucleolin; the nuclei are counterstained with a DNA-intercalating dye. Figure 4B shows a parallel phase contrast micrograph. Figures 4C and 4D are 15 duplicates of Figures 4A and 4B, except markings have been added to better indicate areas of staining. In region 1, the signal is strong on the cells (faint signal in relation to the nuclear staining in Fig. 4A); these cells are in loosely-organized tissue and are less densely-packed, suggesting that they are malignant. In region 2, normal cells (as delineated by well-packed cells and organized tissue), cells display no plasma membrane nucleolin signal.

20

Example 4 Plasma membrane nucleolin expression in lung carcinoma cells

This example demonstrates that lung carcinoma cells can be easily identified by staining for plasma membrane nucleolin.

25 NCI-H1299 (non-small cell lung carcinoma isolated from *H. sapiens* lymph node; (Giaccone *et al.*, 1992; Lin and Chang, 1996)) and NCI-H82 (small cell lung carcinoma cells, *H. sapiens*, (Carney *et al.*, 1985; Little *et al.*, 1983; Takahashi *et al.*, 1989)) cells were released from culture substrates with trypsin, resuspended into single cells and plated onto microscope slides. The cells were incubated at 37° C until 30 they were well-attached as assayed by visual inspection using a microscope. After rinsing the cells once with PBS for 2 minutes, they were fixed in 4% formaldehyde/PBS for at least 15 minutes at 22° C. After washing twice with PBS, 5

5 minutes/wash, non-specific binding sites were blocked for 15-60 minutes with 1% NGS/PBS at 22°C, and then incubated with mouse anti-nucleolin antibodies for 1 hour to overnight at 4°C. The samples were washed four times, 5 minutes each with PBS and then incubated with goat anti-mouse pAb labeled with FITC-labeled 5 secondary antibodies diluted in PBS with propidium iodide (to stain nuclei) for 1 hour at 22°C. After again washing four times with PBS for 5 minutes each, the samples were mounted in Mowiol mounting media, observed under a microscope and photographed.

10 Figure 5 shows whole cells probed for plasma membrane nucleolin of the two lung cancer cell lines, NCI-H82 (Figure 5A; a parallel phase contrast image is shown in 5B) and NCI-H1299 (Figure 5C; a parallel phase contrast image is shown in 5D). In both cell lines, plasma membrane nucleolin staining is strong; examples of well-stained cells are denoted by asterisk (*) in Figures 5A and 5C.

15 *Example 5 Plasma membrane nucleolin staining of clinical specimens*

To test the feasibility of using this novel method of assaying plasma membrane nucleolin to diagnose tumor, pre-malignant and malignant cells, clinical specimens from healthy subjects and those suffering from a cancer were collected. Samples from peripheral blood, bone marrow and tumor biopsy samples were 20 obtained and stained for plasma membrane nucleolin as described in Example 4. Figure 6 shows phase contrast (B, D, F) and immunofluorescent images (A, C, E) of peripheral blood (A, B) or bone marrow (C, D and E, F). Highly stained cells for plasma membrane nucleolin are marked with an asterisk (*); these were only seen in those patients suffering from carcinomas (A,B and C,D), while cells from a healthy 25 patient did not display any plasma membrane staining (E, F).

Example 6 Inhibition of tumor growth

A SCID mouse colony was developed using original SCID mice (C.B-17/1erACSCID) obtained from Taconic (Germantown, NY). The mice were housed 30 in microisolator cages (Allentown Caging Equipment Company, Allentown, New Jersey) and maintained under specific pathogen-free conditions. The mice ate NIH31 irradiated pellets (Tekland Premier; Madison, WI) and drank autoclaved water. Mice

were screened monthly by ELISA serology for mycoplasma, mouse hepatitis virus, pinworms, and Sendai virus. They tested negative.

5 Female mice 6-8 weeks of age were bled (200 μ l) by retro-orbital puncture in order to screen for the presence of mouse immunoglobulin (Ig) using ELISA. Only mice with IgG levels < 20 μ g/ml were used for the experiments. Mice were weighed once weekly. Tumor cell injections were given SC on the mouse's lower right flank in a total volume of 200 μ l. Drug injections were administered by intraperitoneal (IP) injection (200 μ l) when tumors were established (Day 6). As tumors developed, SC tumors were measured for tumor volume estimation (mm^3) in accordance with the formula ($a^2 \times b/2$) where a is the smallest diameter and b is the largest diameter. The 10 mice were sacrificed by CO_2 and tumors were harvested. Harvested tumors were sliced into 3 mm sections, set in 10% neutral buffered formalin for 24 hours, then placed in 70% ethanol, and embedded in paraffin blocks.

15 The MDA-MB-231 breast cell line was grown in HyQ RPMI-1640 (1X) media (HyClone, Logan, UT) with 2.05 mM L-glutamine supplemented with 10% fetal bovine serum (Sigma; St. Louis, MO), and maintained in 5% CO_2 -95% air humidified atmosphere at 37°C. One flask of sub-confluent cells was harvested using 0.25% trypsin-EDTA (HyClone; Logan, UT) and were counted using the trypan blue assay technique. The other flasks of sub-confluent cells were scraped. Cells (95- 20 100% viability) were re-suspended respectively at a concentration of 8×10^6 cells /200 μ l of sterile saline.

25 Taxol 10 mg/kg was prepared and administered IP in the volume of 200 μ l every other day for a total of 5 injections in less than an hour from the preparation time. The monoclonal and polyclonal antibodies, mouse IgG, and poly rabbit IgG were prepared and injected within an hour IP in the volume of 200 μ l for the initial loading dose of 10 mg/kg. The remaining amount of antibodies were prepared at a maintenance dose of 3 mg/kg in 200 μ l, which was aliquoted and frozen into 6 separate tubes per antibody for weekly injections for a total of 6 weeks. Each weekly maintenance dose was thawed and injected within an hour. The PBS 1x control was 30 administered IP in 200 μ l weekly for a total of 7 injections. Results of the experiment demonstrate significantly greater tumor regression with the combination of any antibody composition and Taxol.

Example 7 (Prophetic) Correlation of the degree of plasma membrane nucleolin expression and cancer aggressiveness

5 Thirty-three lung carcinoma cell lines are analyzed, mostly available from the American Type Culture Collection (Manassas, VA). Cell doubling time is calculated by determining cell density at regular intervals using the MTT assay and confirmed by counting the cells using trypan blue exclusion. In each experiment HeLa cells (Gey *et al.*, 1952) are included as an internal control. Each value is determined from at least two independent experiments with triplicate samples. To determine levels of 10 nuclear and plasma membrane nucleolin, two methods are implemented. First, nuclear and plasma membrane extracts are prepared from each cell line using methods that have as described (Ausubel *et al.*, 1987; Bates *et al.*, 1999; Yao *et al.*, 1996a). Briefly, cells are harvested and resuspended in a hypotonic buffer, then allowed to 15 swell on ice for several minutes. Cells are lysed using a Dounce homogenizer, and nuclei are collected by centrifugation. Nuclei are resuspended in a high salt buffer to extract nuclear proteins; salt is then removed by dialysis. Plasma membrane proteins can be isolated from the S-100 fraction and are separated from cytosolic proteins and other organelles by centrifugation through a sucrose gradient. Nuclear and PM extracts from different cell are analyzed by Western blot analysis (Ausubel *et al.*, 1987) using an anti-nucleolin antibody (Santa Cruz) followed by chemiluminescent 20 visualization. Nucleolin levels are then quantified by densitometry of the resulting signal recorded on X-ray film and normalized to the intensity of HeLa extract controls. The second approach to determine nucleolin levels involves immunofluorescent probing of the cell lines for nucleolin. Cells are probed for 25 nucleolin surface expression in parallel with DU145 cells (Mickey *et al.*, 1977; Stone *et al.*, 1978) as a positive control, HS27 cells as a negative control and HeLa cells as a reference (see Figure 2). Cells are photographed and ranked in order of degree of signal, which may also be quantified (using systems that use software and images to quantitate pixels; in this instance, video images are used) or qualitatively evaluated. 30 The data are then subjected to statistical analysis to demonstrate correlations with the degree of cell proliferation (higher rates of cell proliferation indicate more aggressive

cancer cells) with the intensity of nucleolin signal across the entire sample and within subsets.

Example 8 (Prophetic) Lung cancer detection

5 In this example, patient biopsies, sputum samples and resected lung tissue are probed for plasma membrane nucleolin, and these results are compared to other diagnostic and prognostic markers for lung cancer, utilizing archival and routine clinical specimens for this study.

Methods

10 Specimens including bronchial biopsies, sputum samples, and resected lung tissue are obtained from human subjects, both healthy and those suffering from lung cancer, and each sample encoded such that at the time of nucleolin probing and observation, the sample origin is unknown.

15 Probing these samples using immunohistochemical techniques are then implemented. For example, plasma membrane nucleolin is probed with one or more anti-nucleolin Abs selected from Table 1, a signal generated from a fluorophore-tagged secondary Ab, and the samples observed and photographed. Appropriate controls include probing with the secondary antibody only, probing with no antibodies, probing with pre-immune serum only, and probing with an antibody known not to react with the cell types being analyzed. To facilitate visualization and localization determination, the cells can be counterstained with Hoechst 33258 or propidium iodide (to visualize nuclei) and/or with fluorescent-tagged phalloidin or phalloidin (to visualize the actin cytoskeleton). The samples are observed, scored (surface signal indicating plasma membrane nucleolin expression) and documented.

20
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30 All cited publications are incorporated herein by reference. The terms and expressions which have been employed in the present disclosure are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof; various modifications are possible and are within the scope of the invention.

REFERENCES

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1987. Current protocols in molecular biology. John Wiley & Sons, New York.

Babkes, M., A. Ranford, and K. Yaeger. 2001. Sputum trap manifold with nested caps/ US Patent 6,325,785.

5 Bandman, O., H. Yue, N. Corley, and P. Shah. 1999. Human nucleolin-like protein/ US Patent No. 5,932,475.

Bates, P.J., J.B. Kahlon, S.D. Thomas, J.O. Trent, and D.M. Miller. 1999. Antiproliferative activity of G-rich oligonucleotides correlates with protein binding. *J Biol Chem.* 274:26369-77.

10 Beach, D., E. Bernstein, A. Caudy, S. Hammond, and G. Hannon. 2001. Methods and compositions for RNA interference/ WO 01/68836.

Byrne, C.J. 1986. Laboratory tests : implications for nursing care. Addison-Wesley Pub. Co. Health Sciences Division, Menlo Park, Calif. xxi, 756 pp.

15 Callebaut, C., J. Blanco, N. Benkirane, B. Krust, E. Jacotot, G. Guichard, N. Seddiki, J. Svab, E. Dam, S. Muller, J.P. Briand, and A.G. Hovanessian. 1998. Identification of V3 loop-binding proteins as potential receptors implicated in the binding of HIV particles to CD4(+) cells. *J Biol Chem.* 273:21988-97.

Carney, D.N., A.F. Gazdar, G. Bepler, J.G. Guccion, P.J. Marangos, T.W. Moody, M.H. Zweig, and J.D. Minna. 1985. Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res.* 45:2913-23.

20 Cech, T., A. Zaug, and M. Been. 1992. RNA ribozyme polymerases, dephosphorylases, restriction endoribonucleases and methods/ US 5,093,246.

Cole, S.P., E.H. Vreeken, and J.C. Roder. 1985. Antibody production by human X human hybridomas in serum-free medium. *J Immunol Methods.* 78:271-8.

25 Coligan, J.E. 1996. Current protocols in immunology. Wiley, [New York]. 3 v. (loose leaf) pp.

Davis, K.A., Y. Lin, B. Abrams, and S.D. Jayasena. 1998. Staining of cell surface human CD4 with 2'-F-pyrimidine-containing RNA aptamers for flow cytometry. *Nucleic Acids Res.* 26:3915-24.

Derenzini, M. 2000. The AgNORs. *Micron.* 31:117-20.

30 Fire, A., S. Kostas, M. Montgomery, L. Timmons, S. Xu, H. Tabara, S. Driver, and C. Mello. 2003. Genetic inhibition by double-stranded RNA/ US 6,506,559.

Gey, G., W. Coffman, and M. Kubicek. 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* 12:264.

Giaccone, G., J. Battey, A.F. Gazdar, H. Oie, M. Draoui, and T.W. Moody. 1992. Neuromedin B is present in lung cancer cell lines. *Cancer Res.* 52:2732s-2736s.

Ginisty, H., H. Sicard, B. Roger, and P. Bouvet. 1999. Structure and functions of nucleolin. *J Cell Sci.* 112:761-72.

Goding, J.W. 1996. Monoclonal antibodies: Principles and Practice. Academic Press, San Diego. 492 pp.

5 Harlow, E., and D. Lane. 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 726 pp.

Harlow, E., and D. Lane. 1999. Using antibodies: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

10 Irving, R.A., G. Coia, A. Roberts, S.D. Nuttall, and P.J. Hudson. 2001. Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics. *J Immunol Methods.* 248:31-45.

Jakobovits, A., R. Kucherlapati, S. Klapholz, M. Mendez, and L. Green. 1998. Transgenic mammals having human Ig loci including plural V/ WO 98/24893.

15 Kim, C.S., B.B. Berkley, W.M. Abraham, and A. Wanner. 1982. A micro double capillary method for rheologic measurements of lower airway secretions. *Bull Eur Physiopathol Respir.* 18:915-27.

King, M., and D. Speert. 2002. Use of destran and other polysaccharides to improve mucus clearance/ US Patent 6,339,075.

Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature.* 256:495-7.

20 Koide, S. 2002. Nucleic acids encoding artificial antibody polypeptides/ US 6,462,189.

Langer, P.R., A.A. Waldrop, and D.C. Ward. 1981. Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proc Natl Acad Sci U S A.* 78:6633-7.

LaPosta, V., and J. Eldridge. 2001. Adjuvant and vaccine compositions containing monophosphoryl lipid A/ US 6,306,404.

25 Lin, D.L., and C. Chang. 1996. p53 is a mediator for radiation-repressed human TR2 orphan receptor expression in MCF-7 cells, a new pathway from tumor suppressor to member of the steroid receptor superfamily. *J Biol Chem.* 271:14649-52.

Little, C.D., M.M. Nau, D.N. Carney, A.F. Gazdar, and J.D. Minna. 1983. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature.* 306:194-6.

30 Lonberg, N., and R. Kay. 1998. Transgenic non-human animals capable of producing heterologous antibodies/ US 5,770,429.

McNicol, A.M., and J.A. Richmond. 1998. Optimizing immunohistochemistry: antigen retrieval and signal amplification. *Histopathology.* 32:97-103.

Mickey, D.D., K.R. Stone, H. Wunderli, G.H. Mickey, R.T. Vollmer, and D.F. Paulson. 1977. Heterotransplantation of a human prostatic adenocarcinoma cell line in nude mice. *Cancer Res.* 37:4049-58.

Miller, D., P. Bates, and J. Trent. 2000. Antiproliferative activity of G-rich oligonucleotides and method of using same to bind to nucleolin/ WO 00/61597.

5 Mitchell, L. 1997. Therapeutic molecules generated by trans-splicing/ WO 09/722250.

Naito, M., H. Hamada, and T. Tsuruo. 1988. ATP/Mg²⁺-dependent binding of vincristine to the plasma membrane of multidrug-resistant K562 cells. *J Biol Chem.* 263:11887-91.

Orfao, A., and A. Ruiz-Arguelles. 1996. General concepts about cell sorting techniques. *Clin Biochem.* 10 29:5-9.

Robinson, J.M., and D.D. Vandre. 2001. Antigen retrieval in cells and tissues: enhancement with sodium dodecyl sulfate. *Histochem Cell Biol.* 116:119-30.

Rubin, B., and M. Newhouse. 1999. Use of surface active agents to promote mucus clearance/ US Patent 5,925,334.

15 Sharon, J. 1995. Polyclonal antibody libraries/ WO 95/20401.

Shi, S.R., R.J. Cote, and C.R. Taylor. 1997. Antigen retrieval immunohistochemistry: past, present, and future. *J Histochem Cytochem.* 45:327-43.

Shi, S.R., R.J. Cote, and C.R. Taylor. 2001. Antigen retrieval techniques: current perspectives. *J Histochem Cytochem.* 49:931-7.

20 Singh, S., and P. Dias. 2002. Transgenic avian species for making human and chimeric antibodies/ US 2002/0028488.

Sorokina, E.A., and J.G. Kleinman. 1999. Cloning and preliminary characterization of a calcium-binding protein closely related to nucleolin on the apical surface of inner medullary collecting duct cells. *J Biol Chem.* 274:27491-6.

25 Srivastava, M., and H.B. Pollard. 1999. Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. *Faseb J.* 13:1911-22.

Stedman, T.L. 2000. Stedman's medical dictionary. Lippincott Williams & Wilkins, Philadelphia. xxxvi, [127], 2098 p. pp.

Stone, K.R., D.D. Mickey, H. Wunderli, G.H. Mickey, and D.F. Paulson. 1978. Isolation of a human prostate carcinoma cell line (DU 145). *Int J Cancer.* 21:274-81.

30 Surani, A., M. Neuberger, and M. Bruggemann. 1996. Production of antibodies from transgenic animals/ US 5,545,807.

Takahashi, T., M.M. Nau, I. Chiba, M.J. Birrer, R.K. Rosenberg, M. Vinocour, M. Leviit, H. Pass, A.F. Gazdar, and J.D. Minna. 1989. p53: a frequent target for genetic abnormalities in lung cancer. *Science*. 246:491-4.

Tuschl, T., S. Elbashir, and W. Lendeckel. 2002. RNA interference mediating small RNA molecules/ 5 WO 02/44,321.

Tuschl, T., P. Sharp, P. Zamore, and D. Bartel. 2001. RNA sequence-specific mediators of RNA interference/ WO 01/075,164.

Tuteja, R., and N. Tuteja. 1998. Nucleolin: a multifunctional major nucleolar phosphoprotein. *Crit Rev Biochem Mol Biol.* 33:407-36.

10 van de Loosdrecht, A.A., R.H. Beelen, G.J. Ossenkoppele, M.G. Broekhoven, and M.M. Langenhuijsen. 1994. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *J Immunol Methods.* 174:311-20.

15 Wysocki, L.J., and V.L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc Natl Acad Sci U S A.* 75:2844-8.

Xu, X., F. Hamhoutia, S.D. Thomas, T.J. Burke, A.C. Girvan, W.G. McGregor, J.O. Trent, D.M. Miller, and P.J. Bates. 2001. Inhibition of DNA Replication and Induction of S Phase Cell Cycle Arrest by G-rich Oligonucleotides. *J Biol Chem.* 276:43221-30.

20 Yao, G.Q., S. Corrias, and Y.C. Cheng. 1996a. Identification of two oligodeoxyribonucleotide binding proteins on plasma membranes of human cell lines. *Biochem Pharmacol.* 51:431-6.

Yao, G.Q., S. Corrias, and Y.C. Cheng. 1996b. Identification of two oligodeoxyribonucleotide binding proteins on plasma membranes of human cell lines. *Biochem Pharmacol.* 51:431-6.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of treating cancer in a mammal comprising administering to said mammal a therapeutically effective amount of an anti-nucleolin agent and a pharmaceutically acceptable carrier, wherein the anti-nucleolin agent comprises an anti-nucleolin antibody.

5 2. The method of claim 1, wherein said anti-nucleolin antibody is substantially non-immunogenic to humans.

10 3. The method of claim 1 or claim 2, further comprising administering to said mammal a chemotoxic or chemotherapeutic agent.

15 4. The method of any one of claims 1 to 3, further comprising treating said mammal with radiation therapy.

5 5. The method of claim 3, wherein said chemotoxic or chemotherapeutic agent is selected from the group consisting of cyclophosphamide, etoposide, doxorubicin, methotrexate, vincristine, procabazine, prednizone, dexamethasone, 20 tamoxifen citrate, carboplatin, cisplatin, oxaliplatin, 5-fluorouracil, camptothecin, zoledronic acid, ibandronate and mytomycin.

6. Use of an anti-nucleolin agent in the manufacture of a medicament for treating cancer in a mammal, wherein the anti-nucleolin agent comprises an anti-nucleolin antibody.

25 7. Use of claim 6, wherein said anti-nucleolin antibody is substantially non-immunogenic to humans.

30 8. Use of claim 6 or claim 7, wherein the medicament is for treating a mammal being treated with a chemotoxic or chemotherapeutic agent.

9. Use of any one of claims 6 to 8, wherein the medicament is for treating a mammal being treated with radiation therapy.

10. Use of claim 8, wherein said chemotoxic or chemotherapeutic agent is selected from the group consisting of cyclophosphamide, etoposide, doxorubicin, methotrexate, vincristine, procabazine, prednizone, dexamethasone, tamoxifen citrate, carboplatin, cisplatin, oxaliplatin, 5-fluorouracil, camptothecin, zoledronic acid, ibandronate and mytomycin.

11. A method according to claim 1, or use according to claim 6, substantially as hereinbefore described with reference to any one of the examples or figures.

Figure 1

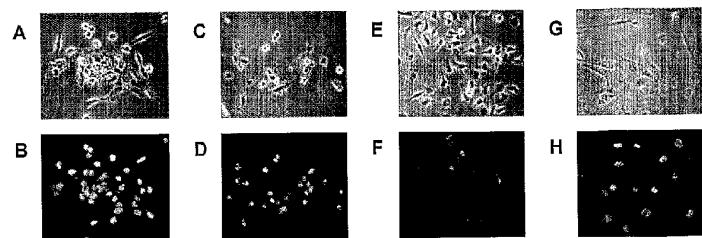


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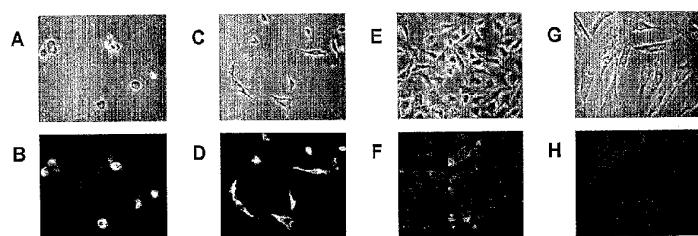


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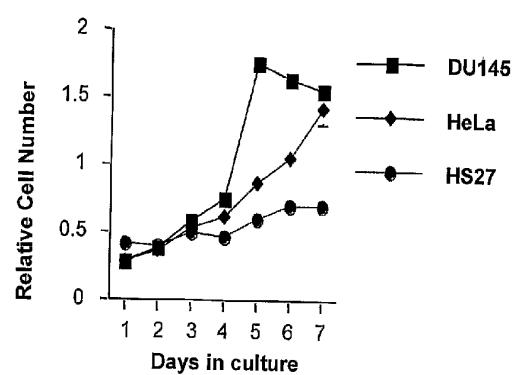


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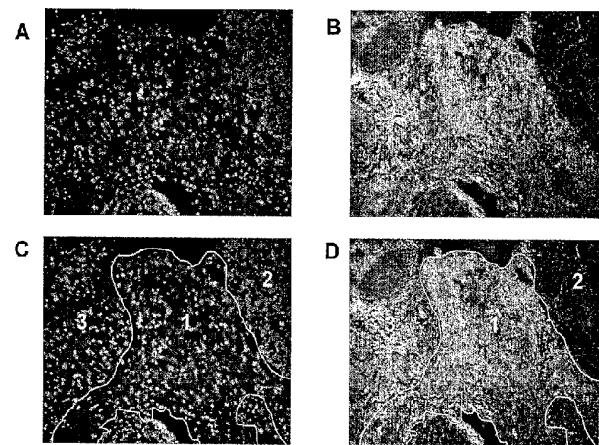
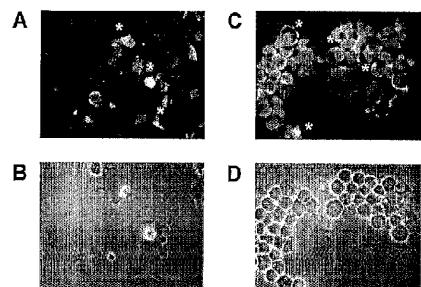
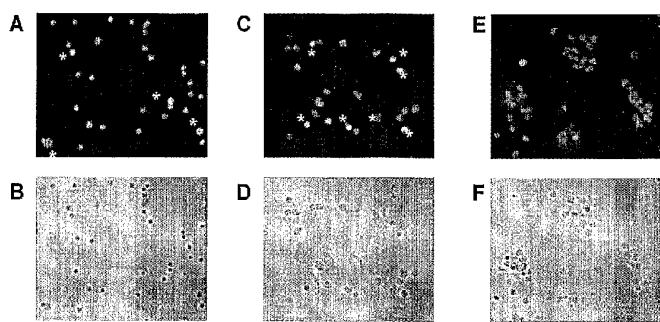


Figure 5



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Figure 6

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PTCTUS2004033174 LOU01-012-CIP-WO SEQUENCE LISTING from US case disk
polynucleotide sequence

<400> 4
tttgggtggtg gtgggttggg tgggtgggg 29

<210> 5
<211> 13
<212> DNA
<213> Artificial sequence
<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 5
tgggtgggtt ggt 13

<210> 6
<211> 14
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 6
ggtgtggtg gtgg 14

<210> 7
<211> 15
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 7
gttgtttgggg tggtt 15

<210> 8
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<212> DNA
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<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 8
ttgggggggg tgggt 15

<210> 9
<211> 25
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 9
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25

<210> 10
<211> 26
<212> DNA
<213> Artificial sequence
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polynucleotide sequence
<400> 10
ggtgtggtg gttgtggg tggtgg 26

<210> 11
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<212> DNA
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<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence
<400> 11
tttgggtggtg gtgggtgtgg tggtggtg 28

<210> 12
<211> 28
<212> DNA
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<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence
<400> 12
tttgggtggtg gtgggtgtgg ggtgggtgg 28

<210> 13
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<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence
<400> 13
ggtgtggtg gttgtgggg tggtggttt 29

<210> 14
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<212> DNA
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<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence
<400> 14
ggtgtggtg gtgggtgtgg tggtgggtt gg 32

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PCTUS2004033174 LOU01-012-CIP-WO SEQUENCE LISTING from US case disk

<210> 15
<211> 32
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<400> 15
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<210> 16
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<212> DNA
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<210> 17
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<212> DNA
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<210> 18
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<212> DNA
<213> Artificial Sequence
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<400> 18
tttcctcctc ctcccttcctcc tcctcc 29

<210> 19
<211> 24
<212> DNA
<213> Artificial Sequence
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<223> Description of Artificial Sequence: Synthetic polynucleotide sequence
<400> 19
tttagggtag ggtaggggtt aggg 24

<210> 20
<211> 11
<212> DNA

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PCTUS2004033174 LOU01-012-CIP-WO SEQUENCE LISTING from US case disk

<213> Artificial sequence

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<223> Description of Artificial Sequence: Synthetic polynucleotide sequence

<400> 20
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11

<210> 21
<211> 14
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic polynucleotide sequence

<400> 21
ggtgttgtg gtgg

14

<210> 22
<211> 15
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic polynucleotide sequence

<400> 22
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15

<210> 23
<211> 10
<212> DNA
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<220>

<223> Description of Artificial sequence: Synthetic polynucleotide sequence

<400> 23
gggttttggg

10

<210> 24
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic polynucleotide sequence

<400> 24
ggtttttgtt ttggttttgg

20

<210> 25
<211> 15
<212> DNA
<213> Artificial Sequence

<220>

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PCTUS2004033174 LOU01-012-CIP-WO SEQUENCE LISTING from US case disk
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 25
ggttggtg gttgg 15

<210> 26
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 26
ggggtttgg gg 12

<210> 27
<211> 10
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<213> Artificial Sequence

<220>
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polynucleotide sequence

<400> 27
gggttttggg 10

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

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 28
ggggtttgg ggttttggg ttttggg 28

<210> 29
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 29
ttggggttgg ggttggggtt gggg 24

<210> 30
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
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polynucleotide sequence

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PCTUS2004033174 LOU01-012-CIP-WO SEQUENCE LISTING from US case disk
<400> 30
gggtgggtgg gtgggt 16

<210> 31
<211> 26
<212> DNA
<213> Artificial sequence

<220>
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polynucleotide sequence

<400> 31
ggttttgggtt ttgggtttgg ttttgg 26

<210> 32
<211> 29
<212> DNA
<213> Artificial sequence

<220>
<223> Description of Artificial sequence: Synthetic
polynucleotide sequence

<400> 32
tttcctccctc ctccttctcc tcctccctcc 29

<210> 33
<211> 26
<212> DNA
<213> Artificial sequence

<220>
<223> Description of Artificial sequence: Synthetic
polynucleotide sequence

<400> 33
cctccctccctc cttctccctcc tcctcc 26

<210> 34
<211> 6
<212> DNA
<213> Artificial sequence

<220>
<223> Description of Artificial sequence: Synthetic
polynucleotide sequence

<400> 34
tgggggt 6

<210> 35
<211> 7
<212> DNA
<213> Artificial sequence

<220>
<223> Description of Artificial sequence: Synthetic
polynucleotide sequence

<400> 35
gcatgct 7

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<210> 36
<211> 11
<212> DNA
<213> Artificial Sequence

<220>
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polynucleotide sequence

<400> 36
gcggtttgcg g 11

<210> 37
<211> 4
<212> DNA
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<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 37
tagg 4

<210> 38
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
polynucleotide sequence

<400> 38
gggggttttttggg tgtggggtttggg 23