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(54) AMPLIFICATION OF CYP24 AND USES THEREOF

AMPLIFIZIERUNG VON CYP24 UND ANWENDUNG DAFÜR

AMPLIFICATION DU GENE CYP24 ET APPLICATIONS DE CELUI-CI

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- **TANNER M M ET AL: "AMPLIFICATION OF CHROMOSOMAL REGION 20Q13 IN INVASIVE BREAST CANCER: PROGNOSTIC IMPLICATIONS" CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 1, no. 12, December 1995 (1995-12), pages 1455-1461, XP001109054 ISSN: 1078-0432**
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Description**FIELD OF THE INVENTION**

5 [0001] This invention pertains to the field of cancer genetics and cytogenetics. In particular, this invention pertains to the identification of an association between amplification(s) of the *CYP24* gene and cancer.

BACKGROUND OF THE INVENTION

10 [0002] Chromosome abnormalities are often associated with genetic disorders, degenerative diseases, and cancer. The deletion or multiplication of copies of whole chromosomes and the deletion or amplifications of chromosomal segments or specific regions are common occurrences in cancer (Smith (1991) *Breast Cancer Res. Treat.* 18: Suppl. 1: 5-14; van de Vijer(1991) *Biochim. Biophys. Acta.* 1072:33-50). In fact, amplifications and deletions of DNA sequences can be the cause of a cancer. For example, proto-oncogenes and tumor-suppressor genes, respectively, are frequently
15 characteristic of tumorigenesis (Dutrillaux (1990) *Cancer Genet. Cytogenet.* 49: 203-217). Clearly, the identification and cloning of specific genomic regions associated with cancer is crucial both to the study of tumorigenesis and in developing better means of diagnosis and prognosis.

[0003] Studies using comparative genomic hybridization (CGH) have revealed approximately twenty amplified genomic regions in human breast tumors (Muleris (1994) *Genes Chromosomes Cancer* 10:160-170; Kalliioniemi (1994) *Proc. Natl. Acad. Sci. USA* 91: 2156-2160; Isola (1995) *Am. J. Pathol.* 147:905-911). These regions are predicted to encode dominantly acting genes that may play a role in tumor progression or response to therapy. Three of these amplified regions have been associated with established oncogenes: ERBB2 at 17q 12, MYC at 8q24 and CCND1 and EMS1 at 11q13. In breast cancer, ERBB2 and CCND1/EMS1 amplification and overexpression are associated with decreased life expectancy (Gaudray (1992) *Mutat. Res.* 276:317-328; Borg (1991) *Oncogene* 6:137-143). MYC amplification has
20 been associated with lymph node involvement, advanced stage cancer and an increased rate of relapse (Borg (1992) *Intern. J. Cancer* 51: 687-691; Beams (1995) *Gene* 159: 11-18). Clearly, the identification of additional amplified genomic regions associated with breast cancer or other tumor cells is critical to the study of tumorigenesis and in the development of cancer diagnostics.

[0004] One of the amplified regions found in the CGH studies was on chromosome 20, specifically, 20q13. Amplification of 20q13 was subsequently found to occur in a variety of tumor types and to be associated with aggressive tumor behavior. Increased 20q 13 copy number was found in 40% of breast cancer cell lines and 18% of primary breast tumors (Kalliioniemi (1994) *supra*). Copy number gains at 20q13 have also been reported in greater than 25% of cancers of the ovary (Iwabuchi (1995) *Cancer Res.* 55:6172-6180), colon (Schlegel (1995) *Cancer Res.* 55: 6002-6005), head-and-neck (Bockmuhl (1996) *Laryngor.* 75: 408-414), brain (Mohapatra (1995) *Genes Chromosomes Cancer* 13: 86-93), and pancreas (Solinas-Toldo (1996) *Genes Chromosomes Cancer* 20:399-407).
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[0005] The 20q13 region was analyzed at higher resolution in breast tumors and cell lines using fluorescent in situ hybridization (FISH). A 1.5 megabase (Mb) wide amplified region within 20q13 was identified (Stokke (1995) *Genomics* 26: 134-137; Tanner (1994) *Cancer Res.* 54:4257- 4260). Interphase FISH revealed low-level (>1.5X) and high level (>3X) 20q13 sequence amplification in 29% and 7% of breast cancers, respectively (Tanner (1995) *Clin. Cancer Res.* 1: 1455-1461). High level amplification was associated with an aggressive tumor phenotype (Tanner (1995) *supra*; Courjal (1996) *Br. J. Cancer* 74: 1984). Another study, using FISH to analyze 14 loci along chromosome 20q in 146 uncultured breast carcinomas, identified three independently amplified regions, including RMC20C001 region at 20q 13.2 (highly amplified in 9.6% of the cases), PTPN1 region 3 Mb proximal (6.2%), and AIB3 region at 20q11 (6.2%) (Tanner (1996) *Cancer Res.* 56:3441-3445). Clearly, definitive characterization of amplified regions within 20q13 would be an important step in the diagnosis and prognosis of these cancers.
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[0006] Increased copy number of chromosome 20q in cultured cells also has been associated with phenotypes characteristic of progressing tumors, including immortalization and genomic instability. For example, increased copy number at 20q11-qter has been observed frequently in human uro-epithelial cells (HUC) (Reznikoff (1994) *Genes Dev.* 8: 2227-2240) and keratinocytes (Solinas-Toldo (1997) *Proc. Natl. Acad. Sci. USA* 94:3854-3859) after transfection with human papilloma virus (HPV)16 E7 or HPV16, respectively. In addition, increased copy number at 20q13.2 has been associated with p53 independent genomic instability in some HPV16 E7 transfected HUC lines (Savelieva (1997) *Oncogene* 14: 551-560). These studies suggest that increased expression of one or more genes on 20q and especially at 20q 13.2 contribute to the evolution of breast cancer and other solid tumors. Several candidate oncogenes have been identified as amplified on 20q, including AIB1 (Anzick (1997) *Science* 277: 965-968), BTAK (Sen (1997) *Oncogene* 14: 2195-200), CAS (Brinkmann (1996) *Genome Res.* 6: 187-194) and TFAP2C (Williamson (1996) *Genomics* 35:262-264). Clearly, definitive characterization of nucleic acid sequences in 20q13 associated with tumor phenotypes would be an important step in the diagnosis and prognosis of these cancers. The present invention fulfills these and other needs.
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SUMMARY OF THE INVENTION

[0007] This invention pertains to the discovery that an amplification of the *CYP24* gene or an increase in *CYP24* activity is a marker for the presence of, progression of, or predisposition to, a cancer (e.g., breast cancer). Using this information, this invention provides methods of detecting/evaluating a predisposition to, progression of, or prognosis of cancer in an animal. Thus, in one embodiment, this invention provides methods of detecting a predisposition to cancer in an animal. The methods involve using a biological sample from said animal, detecting the level of *CYP24* within the biological sample; and comparing the level of *CYP24* with a level of *CYP24* in a control sample taken from a normal, cancer-free tissue;

where an increased level of *CYP24* in the biological sample compared to the level of *CYP24* in the control sample indicates the presence of a cancer in the animal. Similarly, an increased level of *CYP24* in the sample can indicate a poor prognosis for an animal/patient known to have cancer, and/or a reduced survival expectancy, and/or the actual presence of a cancer.

[0008] In one embodiment, the level of *CYP24* is detected by determining the copy number of *CYP24* genes in the cells of the biological sample. In a particularly preferred embodiment, the copy number is measured using Comparative Genomic Hybridization (CGH). In another preferred embodiment, the copy number is determined by hybridization to an array of nucleic acid probes and in another particularly preferred embodiment, the Comparative Genomic Hybridization is performed on an array.

[0009] In another embodiment, the level of *CYP24* is detected by measuring the level of *CYP24* mRNA in the biological sample (e.g., by hybridization to one or more probes in an array), wherein an increased level of *CYP24* RNA in said sample compared to *CYP24* RNA in said control sample indicates a predisposition to cancer. In preferred embodiments, the level of *CYP24* is measured in said biological sample and said control sample at the same vitamin D receptor activity or the *CYP24* levels are normalized to the level of vitamin D receptor activity in the sample and control.

[0010] In still another embodiment, the level of *CYP24* is detected by measuring the level of *CYP24* protein in the biological sample, where an increased level of *CYP24* protein in the sample as compared to *CYP24* protein in said control sample indicates a predisposition to cancer. In preferred embodiments, the level of *CYP24* protein is measured in the biological sample and the control sample at the same vitamin D receptor activity or the protein levels are normalized to the level of vitamin D receptor activity in the sample and control.

[0011] In still yet another embodiment, the level of *CYP24* is detected by measuring the level of 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in the biological sample, wherein an increased level of 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in the sample as compared to 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in the control sample indicates a predisposition to cancer. In preferred methods, the level of 25-hydroxyvitamin D3 24-hydroxylase activity is measured in the biological sample and the control sample at the same vitamin D receptor activity or the activity levels are normalized to the level of vitamin D receptor activity in the sample and control.

[0012] In the methods described herein, the animal(s) are mammals, more preferably mammals selected from the group of humans, non-human primates, canines, felines, murines, bovines, equines, porcines, and lagomorphs.

[0013] Preferred biological samples are selected from the group consisting of excised tissue (e.g., tissue biopsy), whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, and urine.

[0014] In preferred embodiments, the difference between the increased level of *CYP24* in the biological sample and the level of *CYP24* in said control sample is a statistically significant difference (e.g. the increased level of *CYP24* in the biological sample is at least about 2-fold greater, more preferably at least 4-fold greater than the level of *CYP24* in the control sample).

[0015] This invention also provides diagnostic methods of selecting a therapy for treating cancer in an animal. The methods involve performing the assays as described herein (e.g. using a biological sample from said animal; detecting the level of *CYP24* within said biological sample; and comparing said level of *CYP24* with a level of *CYP24* in a control sample from a normal, cancer-free tissue) and selecting a cancer therapy in those animals having an increased level of *CYP24* compared to the level of *CYP24* in said control sample. In preferred embodiments, the cancer therapy is selected from the group consisting of chemotherapy, radiation therapy, surgery, antihormone therapy, and immunotherapy. In some preferred embodiments, the cancer therapy is an adjuvant cancer therapy.

DEFINITIONS

[0016] To facilitate understanding the invention, a number of terms are defined below.

[0017] A "*CYP24* gene" is a DNA sequence that encodes a 25-hydroxyvitamin D3 24-hydroxylase enzyme (see, e.g. GenBank Accession Numbers U60669 S78775 and X59506). The term gene can refer to a mutated copy of the gene, or a fragment of the gene.

[0018] The term "*VDR*" refers to a vitamin D receptor.

[0019] The terms "hybridizing specifically to" and "specific hybridization" and "selectively hybridize to," as used herein

refer to the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence under stringent conditions. The term "stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. A "stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization (e.g., as in array, Southern or Northern hybridizations) are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in, e.g., Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology Hybridization with Nucleic Acid Probes part I, chapt 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier, NY ("Tijssen"). Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on an array or on a filter in a Southern or northern blot is 42°C using standard hybridization solutions (see, e.g., Sambrook (1989) Molecular Cloning: A Laboratory Manual (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, and detailed discussion, below), with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (see, e.g., Sambrook *supra.*) for a description of SSC buffer). A typical stringent wash for an array hybridization is 50% formamide, 2X SSC at 50°C to 50°C. Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example of a low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4x to 6x SSC at 40°C for 15 minutes.

[0020] The term "labeled with a detectable composition", as used herein, refers to a nucleic acid attached to a detectable composition, *i.e.*, a label. The detection can be by, e.g., spectroscopic, photochemical, biochemical, immunochemical, physical or chemical means. For example, useful labels include ^{32}P , ^{35}S , ^3H , ^{14}C , ^{125}I , ^{131}I ; fluorescent dyes (e.g., FITC, rhodamine, lanthanide phosphors, Texas red), electron-dense reagents (e.g. gold), enzymes, e.g., as commonly used in an ELISA (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase), colorimetric labels (e.g. colloidal gold), magnetic labels (e.g. Dynabeads™), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available. The label can be directly incorporated into the nucleic acid, peptide or other target compound to be detected, or it can be attached to a probe or antibody that hybridizes or binds to the target. A peptide can be made detectable by incorporating predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, transcriptional activator polypeptide, metal binding domains, epitope tags). Label can be attached by spacer arms of various lengths to reduce potential steric hindrance or impact on other useful or desired properties (see, e.g., Mansfield (1995) Mol Cell Probes 9: 145-156). It will be appreciated that combinations of labels can also be used. Thus, for example, in some embodiments, different nucleic acids may be labeled with distinguishable (e.g. differently colored) labels.

[0021] The term "nucleic acid" as used herein refers to a deoxyribonucleotide or ribonucleotide in either single- or double-stranded form. The term encompasses nucleic acids, *i.e.*, oligonucleotides, containing known analogues of natural nucleotides which have similar or improved binding properties, for the purposes desired, as the reference nucleic acid. The term also includes nucleic acids which are metabolized in a manner similar to naturally occurring nucleotides or at rates that are improved thereover for the purposes desired. The term also encompasses nucleic-acid-like structures with synthetic backbones. DNA backbone analogues provided by the invention include phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, and peptide nucleic acids (PNAs); see Oligonucleotides and Analogues, a Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) J. Med. Chem. 36:1923-1937; Antisense Research and Applications (1993, CRC Press). PNAs contain non-ionic backbones, such as N-(2-aminoethyl) glycine units. Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197. Other synthetic backbones encompassed by the term include methyl-phosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) Biochemistry 36: 8692-8698), and benzylphosphonate linkages (Samstag (1996) Antisense Nucleic Acid Drug Dev 6: 153-156). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide primer, probe and amplification product.

[0022] The term a "nucleic acid array" as used herein is a plurality of target elements, each target element comprising one or more nucleic acid molecules (probes) immobilized on one or more solid surfaces to which sample nucleic acids can be hybridized. The nucleic acids of a target element can contain sequence(s) from specific genes or clones, e.g. from CYP24. Other target elements will contain, for instance, reference sequences. Target elements of various dimensions can be used in the arrays of the invention. Generally, smaller, target elements are preferred. Typically, a target

element will be less than about 1 cm in diameter. Generally element sizes are from 1 μm to about 3 mm, preferably between about 5 μm and about 1 mm. The target elements of the arrays may be arranged on the solid surface at different densities. The target element densities will depend upon a number of factors, such as the nature of the label, the solid support, and the like. One of skill will recognize that each target element may comprise a mixture of nucleic acids of different lengths and sequences. Thus, for example, a target element may contain more than one copy of a cloned piece of DNA, and each copy may be broken into fragments of different lengths. The length and complexity of the nucleic acid fixed onto the target element is not critical to the invention. One of skill can adjust these factors to provide optimum hybridization and signal production for a given hybridization procedure, and to provide the required resolution among different genes or genomic locations. In various embodiments, target element sequences will have a complexity between about 1 kb and about 1 Mb, between about 10 kb to about 500 kb, between about 200 to about 500 kb, and from about 50 kb to about 150 kb.

[0023] The term "probe" or a "nucleic acid probe", as used herein, is defined to be a collection of one or more nucleic acid fragments whose hybridization to a sample can be detected. The probe may be unlabeled or labeled as described below so that its binding to the target or sample can be detected. Particularly in the case of arrays, either probe or target nucleic acids may be affixed to the array. Whether the array comprises "probe" or "target" nucleic acids will be evident from the context. Similarly, depending on context, either the probe, the target, or both can be labeled. The probe is produced from a source of nucleic acids from one or more particular (preselected) portions of the genome, *e.g.*, one or more clones, an isolated whole chromosome or chromosome fragment, or a collection of polymerase chain reaction (PCR) amplification products. The probes of the present invention are produced from nucleic acids found in the regions described herein. The probe or genomic nucleic acid sample may be processed in some manner, *e.g.*, by blocking or removal of repetitive nucleic acids or enrichment with unique nucleic acids. The word "sample" may be used herein to refer not only to detected nucleic acids, but to the detectable nucleic acids in the form in which they are applied to the target, *e.g.*, with the blocking nucleic acids, etc. The blocking nucleic acid may also be referred to separately. What "probe" refers to specifically is clear from the context in which the word is used. The probe may also be isolated nucleic acids immobilized on a solid surface (*e.g.*, nitrocellulose, glass, quartz, fused silica slides), as in an array. In some embodiments, the probe may be a member of an array of nucleic acids as described, for instance, in WO 96/17958. Techniques capable of producing high density arrays can also be used for this purpose (*see, e.g.*, Fodor (1991) *Science* 767-773; Johnston (1998) *Curr. Biol.* 8: R171-R174; Schummer (1997) *Biotechniques* 23: 1087-1092; Kern (1997) *Biotechniques* 23: 120-124; U.S. Patent No. 5,143,854). One of skill will recognize that the precise sequence of the particular probes described herein can be modified to a certain degree to produce probes that are "substantially identical" to the disclosed probes, but retain the ability to specifically bind to (*i.e.*, hybridize specifically to) the same targets or samples as the probe from which they were derived (*see* discussion above). Such modifications are specifically covered by reference to the individual probes described herein.

[0024] The term "sample of human nucleic acid" as used herein refers to a sample comprising human DNA or RNA in a form suitable for detection by hybridization or amplification. The nucleic acid may be isolated, cloned or amplified; it may be, *e.g.*, genomic DNA, mRNA, or cDNA from a particular chromosome, or selected sequences (*e.g.* particular promoters, genes, amplification or restriction fragments, cDNA, *etc.*) within particular amplicons or deletions disclosed here. The nucleic acid sample may be extracted from particular cells or tissues. The cell or tissue sample from which the nucleic acid sample is prepared is typically taken from a patient suspected of having cancer associated with the amplicon amplification or deletion or translocation being detected. Methods of isolating cell and tissue samples are well known to those of skill in the art and include, but are not limited to, aspirations, tissue sections, needle biopsies, and the like. Frequently the sample will be a "clinical sample" which is a sample derived from a patient, including sections of tissues such as frozen sections or paraffin sections taken for histological purposes. The sample can also be derived from supernatants (of cells) or the cells themselves from cell cultures, cells from tissue culture and other media in which it may be desirable to detect chromosomal abnormalities or determine amplicon copy number. In some cases, the nucleic acids may be amplified using standard techniques such as PCR, prior to the hybridization. The sample may be isolated nucleic acids immobilized on a solid. In one embodiment, the sample may be prepared such that individual nucleic acids remain substantially intact and typically comprises interphase nuclei prepared according to standard techniques.

[0025] The phrase "detecting a cancer" refers to the ascertainment of the presence or absence of cancer in an animal. "Detecting a cancer" can also refer to obtaining indirect evidence regarding the likelihood of the presence of cancerous cells in the animal or to the likelihood or predilection to development of a cancer. Detecting a cancer can be accomplished using the methods of this invention alone, or in combination with other methods or in light of other information regarding the state of health of the animal.

[0026] A "cancer" in an animal refers to the presence of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation, immortality, metastatic potential, rapid growth and proliferation rate, and certain characteristic morphological features. Often, cancer cells will be in the form of a tumor, but such cells may exist alone within an animal, or may be a non-tumorigenic cancer cell, such as a leukemia cell. Cancers include, but are not limited to breast cancer, lung cancer, bronchus cancer, colorectal cancer, prostate cancer, pancreas cancer, stomach cancer,

ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, a melanoma, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, testis cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, and a chondrosarcoma.

5 **[0027]** An "animal" refers to a member of the kingdom Animalia, characterized by multicellularity, the possession of a nervous system, voluntary movement, internal digestion, etc. An "animal" can be a human or other mammal. Preferred animals include humans, non-human primates, and other mammals. Thus, it will be recognized that the methods of this invention contemplate veterinary applications as well as medical applications directed to humans.

10 **[0028]** "Providing a biological sample" means to obtain a biological sample for use in the methods described in this invention. Most often, this will be done by removing a sample of cells from an animal, but can also be accomplished by using previously isolated cells (e.g. isolated by another person), or by performing the methods of the invention *in vivo*.

15 **[0029]** A "biological sample" refers to a cell or population of cells or a quantity of tissue or fluid from an animal. Most often, the sample has been removed from an animal, but the term "biological sample" can also refer to cells or tissue analyzed *in vivo*, i.e. without removal from the animal. Often, a "biological sample" will contain cells from the animal, but the term can also refer to non-cellular biological material, such as non-cellular fractions of blood, saliva, or urine, that can be used to measure *CYP24* levels. Preferred biological samples include tissue biopsies, scrapes (e.g. buccal scrapes), whole blood, plasma, serum, urine, saliva, cell culture, or cerebrospinal fluid.

20 **[0030]** "Detecting a level of *CYP24*" refers to determining the number of *CYP24* genes or the expression level of a gene or genes encoding 25-hydroxyvitamin D3 24-hydroxylase enzyme. The copy number of a gene can be measured in multiple ways known to those of skill in the art, including, but not limited to, Comparative Genomic Hybridization (CGH) and quantitative DNA amplification (e.g. quantitative PCR). Gene expression can be monitored in a variety of ways, including by detecting mRNA levels, protein levels, or protein activity, any of which can be measured using standard techniques. Detection can involve quantification of the level of *CYP24* (e.g. genomic DNA, cDNA, mRNA, protein, or enzyme activity), or, alternatively, can be a qualitative assessment of the level of *CYP24*, in particular in comparison with a control level. The type of level being detected will be clear from the context. Because *CYP24* activity is tightly linked to *VDR* activity, measurement of gene expression is preferably done in combination with a measurement of *VDR* activity.

25 **[0031]** To "compare" levels of *CYP24* means to detect *CYP24* levels in two samples and to determine whether the levels are equal or if one or the other is greater. A comparison can be done between quantified levels, allowing statistical comparison between the two values; or in the absence of quantification, for example using qualitative methods of detection such as visual assessment by a human.

30 **[0032]** A "control sample" refers to a sample of biological material representative of healthy, cancer-free animals, and/or cells or tissues.. The level of *CYP24* in a control sample is desirably typical of the general population of normal, cancer-free animals or of a particular individual at a particular time (e.g. before, during or after a treatment regimen), or in a particular tissue. This sample can be removed from an animal expressly for use in the methods described in this invention, or can be any biological material representative of normal, cancer-free animals, including cancer-free biological material taken from an animal with cancer elsewhere in its body. A control sample can also refer to an established level of *CYP24*, representative of the cancer-free population, that has been previously established based on measurements from normal, cancer-free animals.

35 **[0033]** An "increased level of *CYP24*" means a level of *CYP24*, that, in comparison with a control level of *CYP24*, is detectably higher. The method of comparison can be statistical, using quantified values for the level of *CYP24*, or can be compared using non-statistical means, such as by visual assessment by a human.

40 **[0034]** The "copy number of *CYP24* genes" refers to the number of DNA sequences in a cell encoding a 25-hydroxyvitamin D3 24-hydroxylase enzyme. Generally, for a given gene, an animal has two copies of each gene. The copy number can be increased, however, by gene amplification or duplication, or reduced by deletion.

45 **[0035]** When a level of *CYP24* mRNA, protein, enzyme activity, or copy number is "measured," it is assessed using qualitative or quantitative methods. Preferably, the level is determined using quantitative means, allowing the statistical comparison of values obtained from biological samples and control values. The level can also be determined using qualitative methods, such as the visual analysis and comparison by a human of multiple visibly labeled samples, e.g. fluorescently labeled samples detected using a fluorescent microscope or other optical detector (e.g. image analysis system, etc.). When a level of *CYP24* mRNA, protein, or enzyme activity is measured the measurement preferably includes a measurement of *VDR* activity, and/or a measure of *CYP24* activity in a normal tissue or cell (e.g. from the same animal or from a different "control" animal).

50 **[0036]** "25-hydroxyvitamin D3 24-hydroxylase enzyme activity" means the catalysis of the 24-hydroxylation of 25-hydroxyvitamin D3, 1,α-25 dihydroxyvitamin D3, or other analogous substrates (see, e.g., Stryer (1988) Biochemistry, 3rd Ed., W.H. Freeman and Co.; Jehan et al., (1998) Biochim Biophys Acta 1395:259-265; Seo and Norman (1997) J Bone Miner Res 12:598-606).

55 **[0037]** "Tissue biopsy" refers to the removal of a biological sample for diagnostic analysis. In a patient with cancer,

tissue may be removed from a tumor, allowing the analysis of cells within the tumor.

[0038] When a quantified level of *CYP24* falls outside of a given confidence interval for a normal level of *CYP24*, the difference between the two levels is said to be "statistically significant." If a test value falls outside of a given confidence interval for a normal level of *CYP24*, it is possible to calculate the probability that the test value is truly abnormal and does not just represent a normal deviation from the average. In the methods of this invention, a difference between a test sample and a control can be termed "statistically significant" when the probability of the test sample being abnormal can be any of a number of values, including 0.15, 0.1, 0.05, and 0.01. Numerous sources teach how to assess statistical significance, such as Freund, J.E. (1988) Modern elementary statistics, Prentice-Hall.

[0039] The "survival expectancy" of an animal refers to a prognostic estimate of the outcome of a disease or condition. A "survival expectancy" can refer to a prediction regarding the severity, duration, or progress of a disease, condition, or any symptom thereof. "Survival expectancy" can also refer to the length of time an animal is expected to survive, or to the probability of the animal surviving until a certain time.

[0040] A "method of treating cancer" refers to a procedure or course of action that is designed to reduce or eliminate the number of cancer cells in an animal, or to alleviate the symptoms of a cancer. "A method of treating cancer" does not necessarily mean that the cancer cells will in fact be eliminated, that the number of cells will in fact be reduced, or that the symptoms of a cancer will in fact be alleviated. Often, a method of treating cancer will be performed even with a low likelihood of success, but which, given the medical history and estimated survival expectancy of an animal, is deemed an overall beneficial course of action.

[0041] "Reducing the level of *CYP24* activity" refers to inhibiting the 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in the cell, or lowering the copy number of *CYP24* genes, or decreasing the level of *CYP24* mRNA or protein in the cell (e.g., at a given *VDR* activity level). Preferably, the level of *CYP24* activity is lowered to the level typical of a normal, cancer-free cell, but the level may be reduced to any level that is sufficient to decrease the proliferation of the cell, including to levels below those typical of normal cells.

[0042] "Contacting" a cell with vitamin D is to ensure that the cell is in the presence of vitamin D. In the case of a cell that is not naturally in contact with vitamin D, vitamin D is added to the cell, *in vivo* or *in vitro*. "Vitamin D" refers to any of the family of vitamin D molecules, including but not limited to vitamin D1, vitamin D2, and vitamin D3. It also refers to structural and functional homologs of these molecules, e.g. those that are substrates for the *CYP24* enzyme, as well as metabolic products of vitamin D.

[0043] A "tumor cell" is a cancer cell that is part of a tumor, has been isolated from a tumor, or which is capable of forming a tumor. Tumor cells can exist *in vivo* or *in vitro*.

[0044] A "hyperproliferative cell" is a cell with an abnormally high rate of proliferation, or a cell that proliferates to an abnormally great extent, *i.e.* gives rise to a population of cells that increases in number over time. "Hyperproliferative cells" can exist *in vitro* or *in vivo*.

[0045] An "inhibitor of *CYP24* activity" is a molecule that acts to reduce *CYP24* activity, as defined above. Such inhibitors can include antisense molecules or ribozymes, repressors of *CYP24* gene transcription, or competitive or non-competitive molecular inhibitors of the 25-hydroxyvitamin D3 24-hydroxylase enzyme.

[0046] The phrase "repressor of *CYP24* transcription" refers to a molecule that can prevent the production of *CYP24* mRNA from a *CYP24* gene. Preferably, the molecule binds directly or indirectly to a regulatory element of the *CYP24* gene, thereby preventing the transcription of the *CYP24* gene.

[0047] A "competitive inhibitor of 25-hydroxyvitamin D3 24-hydroxylase" means a molecule that can bind directly or indirectly to a 25-hydroxyvitamin D3 24-hydroxylase enzyme or to its substrate, thereby preventing the binding of the enzyme to its substrate and preventing the 24-hydroxylation of the substrate, *in vitro* or *in vivo*.

[0048] The phrase "non-competitive inhibitor of 25-hydroxyvitamin D3 24-hydroxylase" refers to a molecule that prevents the 24-hydroxylation of a 25-hydroxyvitamin D3 24-hydroxylase enzyme substrate but which does not prevent the binding of the enzyme to the substrate.

[0049] "Screening" for an inhibitor of cell proliferation or of *CYP24* activity means to systematically examine the ability of a population of molecules to inhibit cell proliferation or *CYP24* activity. Screening can be done *in vitro* or *in vivo*. The inhibitory activity of the screened molecules can be assessed directly, e.g. by examining *CYP24* enzyme activity using standard assays, or indirectly, e.g. by monitoring a cellular consequence of *CYP24* enzyme activity, such as cell proliferation.

[0050] A "*CYP24*-expressing cell" is a cell that produces any amount of 25-hydroxyvitamin D3 24-hydroxylase protein. Generally, the amount of enzyme produced by the cell will be detectable using standard techniques.

[0051] A "test agent" is any molecule or non-molecular entity that is tested in a screen. The molecule may be randomly selected for inclusion in the screen, or may be included because of an *a priori* expectation that the molecule will give a positive result in the screen. The molecule may be directly introduced into a cell or a biochemical assay for the purposes of the screen, or it may comprise nucleic acids that encode a polypeptide or RNA that is desirably tested in the screen. Molecules introduced directly into an assay system can include any known chemical or biochemical molecule, including peptides, nucleic acids, carbohydrates, lipids, or any other organic or inorganic molecule. A "test agent" can also refer

to non-molecular entities such as electromagnetic radiation or heat.

[0052] The "proliferation" of a cell refers to the rate at which the cell or population of cells grows and divides, or to the extent to which the cell or population of cells grows, divides, or increases in number. The "proliferation" of a cell can reflect multiple factors including the rate of cell growth and division and the rate of cell death.

5 [0053] The phrase "decreasing the proliferation of a cell" means to reduce the rate or extent of growth or division of a cell or population of cells. Such methods can involve preventing cell division or cell growth, and may also include cell killing, and can be practiced *in vivo* or *in vitro*.

[0054] "CYP24-inhibiting activity" is the ability of a molecule to reduce or prevent the production and/or accumulation of 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in a cell. The molecule can prevent the accumulation at any step of the pathway from the CYP24 gene to enzyme activity, e.g. preventing transcription, reducing mRNA levels, preventing translation, or inhibiting the enzyme itself. The reduction or prevention is preferably ascertained by reference to a control at the same level of VDR activity.

10 [0055] A CYP24 enzyme or CYP24 polypeptide is a protein with 25-hydroxyvitamin D3 24-hydroxylase activity and is most preferably encoded by a CYP24 gene.

15 [0056] The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

[0057] An amino acid, identified by name herein "e.g., arginine" or "arginine residue" as used herein refers to natural, synthetic, or version of the amino acids. Thus, for example, an arginine can also include arginine analogs that offer the same or similar functionality as natural arginine with respect to their ability to be incorporated into a polypeptide, effect folding of that polypeptide and effect interactions of that polypeptide with other polypeptide(s).

BRIEF DESCRIPTION OF THE DRAWINGS

25 [0058]

Figure 1 illustrates Comparative Genomic Hybridization (CGH). In the left panel, total genomic DNAs are isolated from a "test" and a "reference" cell population, labeled with different fluorochromes, and hybridized to normal metaphase chromosomes. Cot-1 DNA is used to suppress hybridization of repetitive sequences. The resulting ratio of the fluorescence intensities of the two fluorochromes at a location on a chromosome is approximately proportional to the ratio of the copy numbers of the corresponding DNA sequences in the test and reference genomes. The right panel shows a similar hybridization to an array of mapped clones. This permits measurement of copy number with resolution determined by the size of the clones and/or the map spacing between them.

30 Figure 2 illustrates a high resolution array CGH measurement on the breast cancer tumor, S21. The test:reference ratios for contiguous target clones from region A at 20q13.2 are plotted with the ratio on each target clone shown as a bar, representing the clone overlaps as determined by STS-content mapping. Clone names have been shortened to the last four digits. The analysis indicates that the overlapping clones, RMC20B4121, RMC20B4087, and RMC20B4195 are the locus of the peak in the copy number profile and that copy number in the region decreases precipitously distal to the overlapping clones, RMC20B4087 and RMC20B4195.

35 Figures 3 illustrates expression of CYP24, VDR and ZNF217 genes in human breast cancer cell lines and tumors with and without induction by 1,25-dihydroxyvitamin D₃ evaluated by RT-PCR. (a) Time course of induction of CYP24 gene expression in MCF7 breast cancer cells incubated with 10⁻⁸ M 1,25-dihydroxyvitamin D₃ and vehicle control (ethanol, EtOH). (b) Gene expression in two breast cancer tumors, S21 and S59. (c) Gene expression in the BT474 cell line. Cells were incubated with 10⁻⁸ M 1,25-dihydroxyvitamin D₃, as described in (a).

DETAILED DESCRIPTION

40 [0059] This invention pertains to the discovery that amplification of the vitamin D 24 hydroxylase (CYP24) gene (GenBank Accession Numbers U60669 S78775 and X59506) occurs in various cancers (e.g., breast tumors). Vitamin D 24 hydroxylase controls activity of the vitamin D system in cells by initiating degradation of the active form of vitamin D3. Without being bound by a particular theory, it is believed that amplification of CYP24 during tumor evolution provides a means to disrupt vitamin D mediated growth control.

45 [0060] Amplification of chromosome band 20q13.2 in human breast cancer is associated with poor prognosis and aggressive tumor behavior (Tanner et al., (1995) Clin. Cancer Res. 1: 1455-1461; Courjal et al. (1996) Br. J. Cancer, 74: 1984-1989), suggesting that overexpression of genes mapping to this region is likely to contribute to the development of breast cancer. Using a new high resolution form of comparative genomic hybridization, array CGH (Pinkel et al. (1998) Nature Genetics, 20: 207-211), we mapped DNA copy number profiles across the region of recurrent amplification at 20q13.2.

[0061] This analysis focused attention on the gene *CYP24*, because it mapped to the narrow genomic interval that is most highly amplified in the most informative tumors and because of existing knowledge of *CYP24* function. *CYP24* encodes vitamin D 24 hydroxylase, an enzyme that catalyzes degradation of the active form of vitamin D, 1,25-dihydroxy-D3 (for reviews, see Walters (1992) *Endocrine Reviews* 13: 719-764; Jones et al. (1998) *Amer. Physiol. Soc.* 78: 1193-1231). Vitamin D is a secosteroid hormone that plays a major role in the regulation of calcium and bone metabolism. However, vitamin D receptors (*VDR*) have also been found in many other so-called "non-classical" tissues not involved in mineral metabolism, including the breast (Berger et al. (1987) *Cancer Res.* 47: 6793-6799; Buras et al. (1994) *Breast Cancer Res. and Treatment* 31: 191-202), indicating a role for vitamin D in these tissues also. Levels of 1,25-dihydroxy-D3 and ligand bound receptor appear to be very tightly controlled in cells by a feedback mechanism. Binding of the hormone to the *VDR* results in activation of *CYP24* transcription to initiate degradation of 1,25-dihydroxy-D3 and inhibition of *CYP1*, the enzyme required for synthesis of 1,25-dihydroxy-D3. In fact, transcription of *CYP24* is so closely coupled to *VDR* levels and activity that activation of transcription from a *CYP24* promoter-reporter construct is used as an assay for *VDR* activity (Arbour et al. (1998) *Anal. Biochem.* 255: 148-154). Thus, without being bound to this theory, we believe the role of *CYP24* in cells is to limit the biological activity of the vitamin D system.

[0062] In the "non-classical" tissues such as breast, vitamin D promotes growth inhibition by directing cells towards differentiation and cessation of proliferation. Breast cancer cells respond to the antiproliferative effects of vitamin D both *in vivo* and *in vitro* (Eisman et al. (1989) *Cancer Res.* 47: 21-25). Breast cancer cell lines generally arrest in the G0/G1 stage of the cell cycle in response to vitamin D, and the MCF-7 breast cancer cell line can be induced to enter apoptosis (Elstner et al. (1995) *Cancer Res.* 55: 2822-2830; Love-Schimenti et al. (1996) *Cancer Res.* 56: 2789-2794; Simboli-Campbell et al. (1997) *Breast Cancer Res. and Treatment*, 42: 31-41). Administration of vitamin D to rodents reduces progression of tumor xenographs (Eisman et al. (1989) *Cancer Res.* 47: 21-25; Colston et al. (1989) *Lancet*, 188-191).

[0063] These growth modulatory properties of vitamin D support the present belief that disruption of the vitamin D system is likely to contribute to neoplasia. This suggestion is further supported by the observation that patients with receptor negative tumors have a poorer prognosis and by epidemiological studies that have established that exposure to sunlight and risk of breast and colon cancer (Gorham et al. (1989) *Can. J. Public Health* 80: 96-100; Gorham et al. (1990) *Int. J. Epidemiol.* 19: 820-824; Garland et al. (1990) *Preventive Medicine* 19: 614-622) are inversely correlated.

[0064] Thus, the present hypothesized oncogenic role of *CYP24* derives from its function to reduce levels of 1,25-dihydroxyvitamin-D3 and so modulate the biological effects of ligand bound *VDR*. This hypothesis is supported by the observation that the antiproliferative activity of vitamin D *in vitro* is enhanced in the presence of hydroxylase inhibitors (Reinhardt and Horst (1989) *Arch. Biochem. Biophys.* 272: 459-465; Zhao et al. (1996) *J. Steroid. Biochem. Mol. Biol.* 57: 197-202). Thus, without being bound by a theory, the present invention is predicated, in part, on the recognition that amplification of *CYP24* abrogates vitamin D mediated growth control by up-regulation of vitamin D degradation in cells, since ligand bound *VDR* will bind to and initiate transcription from an increased number of *CYP24* gene copies.

[0065] In view of these discoveries, *CYP24* provides a good marker for a cancer and/or for the likelihood of (predisposition to) development of a cancer. Thus, in one embodiment, this invention provides methods of detecting the presence of, or a predisposition to, cancer in an animal. The methods involve (i) using a biological sample from an animal (e.g. a human patient); (ii) detecting the level of *CYP24* within the biological sample; and (iii) comparing the level of *CYP24* with a level of *CYP24* in a control sample taken from a normal, cancer-free animal where an increased level of *CYP24* in the biological sample compared to the level of *CYP24* in the control sample indicates the presence of said cancer in said animal. Where the *CYP24* transcript, translated polypeptide, or enzymatic activity is assayed, the methods preferably include a measurement of *VDR* activity and the comparison between sample and control is made at the same *VDR* level or corrections are made reflecting differences in *VDR* level.

[0066] Similarly, the detection of *CYP24* level can also be used to estimate the survival expectancy of an animal with cancer. Because *CYP24* level can be used to assay survival expectancy (e.g. likelihood of progression or recurrence of the disease), an assay of *CYP24* level provides a useful component of a cancer therapy. Thus, in one preferred method of treating cancer, *CYP24* level is assayed and, where it is high relative to the appropriate control or population standard, one or more adjuvant therapies (e.g. radiation therapy, resurgery, chemotherapy, etc.) are selected for the cancer treatment regimen.

[0067] Having identified elevated *CYP24* levels as indicative of a cancer or a predisposition to cancer, *CYP24* level provides a useful target/marker for evaluating potential prophylaxis and/or therapeutics. Thus, for example, the level of *CYP24* activity (at a given level of *VDR* activity) in the presence or absence of one or more putative potential therapeutics or prophylactics provides a measure of the potential activity of the therapeutic/prophylactic compound, i.e., a lower *CYP24* activity in the presence of the compound indicates higher potential activity of the compound.

1. Assays of *CYP24* Level.

[0068] As indicated above, assays of *CYP24* copy number or level of activity (e.g., at a particular vitamin D receptor activity) provide a measure of the presence or likelihood of (predisposition to) a cancer. The sequence of *CYP24* is

known and hence, copy number can be directly measured according to a number of different methods as described below.

[0069] With respect to assays based on *CYP24* "activity" level (e.g., level of transcript, level of translated protein, level of protein enzymatic activity), the close coupling of transcription of *CYP24* to vitamin D receptor (*VDR*) level and activity complicates the evaluation of *CYP24* level. In short, *CYP24* expression levels depend on *VDR* activity as well as the magnitude of transcription resulting from copy number increases. Thus, particularly in embodiments relying on assays of *CYP24* "activity", evaluation of *CYP24* levels preferably involves measurement not only of *CYP24* levels in tumor cells relative to normal tissue, but also the *VDR* levels and activities in the tumors and normal tissues. Such assays are described below.

A) Detection of copy number

[0070] In one embodiment, the presence of, or predilection to cancer, is evaluated simply by a determination of *CYP24* copy number. Methods of evaluating the copy number of a particular gene are well known to those of skill in the art.

1) Hybridization-based assays

[0071] One method for evaluating the copy number of *CYP24*-encoding nucleic acid in a sample involves a Southern transfer. In a Southern Blot, the genomic DNA (typically fragmented and separated on an electrophoretic gel) is hybridized to a probe specific for the target region. Comparison of the intensity of the hybridization signal from the probe for the target region with control probe signal from analysis of normal genomic DNA (e.g., a non-amplified portion of the same or related cell, tissue, organ, etc.) provides an estimate of the relative copy number of the target nucleic acid.

[0072] An alternative means for determining the copy number of *CYP24* is *in situ* hybridization. *In situ* hybridization assays are well known (e.g., Angerer (1987) Meth. Enzymol 152: 649). Generally, *in situ* hybridization comprises the following major steps: (1) fixation of tissue or biological structure to be analyzed; (2) prehybridization treatment of the biological structure to increase accessibility of target DNA, and to reduce nonspecific binding; (3) hybridization of the mixture of nucleic acids to the nucleic acid in the biological structure or tissue; (4) post-hybridization washes to remove nucleic acid fragments not bound in the hybridization and (5) detection of the hybridized nucleic acid fragments. The reagent used in each of these steps and the conditions for use vary depending on the particular application.

[0073] Preferred *hybridization-based assays* include, but are not limited to, traditional "direct probe" methods such as Southern blots or *in situ* hybridization (e.g., FISH), and "comparative probe" methods such as comparative genomic hybridization (CGH). The methods can be used in a wide variety of formats including, but not limited to substrate- (e.g. membrane or glass) bound methods or array-based approaches as described below.

[0074] In a typical *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If a nucleic acid is to be probed, the cells are typically denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled probes specific to the nucleic acid sequence encoding the protein. The targets (e.g., cells) are then typically washed at a predetermined stringency or at an increasing stringency until an appropriate signal to noise ratio is obtained.

[0075] The probes are typically labeled, e.g., with radioisotopes or fluorescent reporters. Preferred probes are sufficiently long so as to specifically hybridize with the target nucleic acid(s) under stringent conditions. The preferred size range is from about 200 bp to about 1000 bases.

[0076] In some applications it is necessary to block the hybridization capacity of repetitive sequences. Thus, in some embodiments, tRNA, human genomic DNA, or Cot-1 DNA is used to block non-specific hybridization.

[0077] In comparative genomic hybridization methods a first collection of (sample) nucleic acids (e.g. from a possible tumor) is labeled with a first label, while a second collection of (control) nucleic acids (e.g. from a healthy cell/tissue) is labeled with a second label. The ratio of hybridization of the nucleic acids is determined by the ratio of the two (first and second) labels binding to each fiber in the array. Where there are chromosomal deletions or multiplications, differences in the ratio of the signals from the two labels will be detected and the ratio will provide a measure of the *CYP24* copy number.

[0078] Hybridization protocols suitable for use with the methods of the invention are described, e.g., in Albertson (1984) EMBO J. 3: 1227-1234; Pinkel (1988) Proc. Natl. Acad. Sci. USA 85: 9138-9142; EPO Pub. No. 430,402; Methods in Molecular Biology, Vol. 33: In Situ Hybridization Protocols, Choo, ed., Humana Press, Totowa, NJ (1994), etc. In one particularly preferred embodiment, the hybridization protocol of Pinkel et al. (1998) Nature Genetics 20: 207-211, or of Kallioniemi (1992) Proc. Natl Acad Sci USA 89:5321-5325 (1992) is used.

2) Amplification-based assays.

[0079] In still another embodiment, amplification-based assays can be used to measure copy number. In such amplification-based assays, the *CYP24* nucleic acid sequences act as a template in an amplification reaction (e.g. Polymer-

ase Chain Reaction (PCR). In a quantitative amplification, the amount of amplification product will be proportional to the amount of template in the original sample. Comparison to appropriate (e.g. healthy tissue) controls provides a measure of the copy number of *CYP24*.

[0080] Methods of "quantitative" amplification are well known to those of skill in the art. For example, quantitative PCR involves simultaneously co-amplifying a known quantity of a control sequence using the same primers. This provides an internal standard that may be used to calibrate the PCR reaction. Detailed protocols for quantitative PCR are provided in Innis et al. (1990) PCR Protocols, A Guide to Methods and Applications, Academic Press, Inc. N.Y.). The known nucleic acid sequence for *CYP24* (see, GenBank Accession Numbers U60669 S78775 and X59506) is sufficient to enable one of skill to routinely select primers to amplify any portion of the gene.

[0081] Other suitable amplification methods include, but are not limited to ligase chain reaction (LCR) (see Wu and Wallace (1989) Genomics 4: 560, Landegren et al. (1988) Science 241: 1077, and Barringer et al. (1990) Gene 89: 117, transcription amplification (Kwoh et al. (1989) Proc. Natl. Acad. Sci. USA 86: 1173), self-sustained sequence replication (Guatelli et al. (1990) Proc. Nat. Acad. Sci. USA 87: 1874), dot PCR, and linker adapter PCR, etc.

B) Detection of gene expression

[0082] As indicated above, *CYP24* level can also be assayed as a marker for a predilection to cancer. However, because of the close coupling of transcription of *CYP24* to vitamin D receptor (*VDR*) level measures of *CYP24* "activity" are preferably coupled with measures of *VDR* activity for use in the assays of this invention. Thus, an elevation of *CYP24* activity, compared to a control at the same level of *VDR* activity, provides an indication of the presence and/or predilection to a cancer.

[0083] In preferred embodiments, *CYP24* activity is characterized by a measure of *CYP24* gene transcript (e.g. mRNA), by a measure of the quantity of translated protein, or by a measure of *CYP24* enzymatic activity (25-hydroxyvitamin D3 24-hydroxylase enzyme activity).

1) Detection of gene transcript.

a) Direct hybridization based assays.

[0084] Methods of detecting and/or quantifying the *CYP24* gene transcript (*CYP24* mRNA or cDNA made therefrom) using nucleic acid hybridization techniques are known to those of skill in the art (see Sambrook *et al. supra*). For example, one method for evaluating the presence, absence, or quantity of *CYP24* cDNA involves a Southern transfer as described above. Briefly, the *CYP24* mRNA is isolated (e.g. using an acid guanidinium-phenol-chloroform extraction method, Sambrook *et al. supra*.) and reverse transcribed to produce cDNA. The cDNA is then optionally digested and run on a gels in buffer and transferred to membranes. Hybridization is then carried out using the nucleic acid probes specific for the target. *CYP24* cDNA.

[0085] The probes can be full length or less than the full length of the nucleic acid sequence encoding the *CYP24* protein. Shorter probes are empirically tested for specificity. Preferably nucleic acid probes are 20 bases or longer in length. (See Sambrook *et al.* for methods of selecting nucleic acid probe sequences for use in nucleic acid hybridization.) Visualization of the hybridized portions allows the qualitative determination of the presence or absence of *CYP24* cDNA.

[0086] Similarly, a Northern transfer may be used for the detection of *CYP24* mRNA directly. In brief, the mRNA is isolated from a given cell sample using, for example, an acid guanidinium-phenol-chloroform extraction method. The mRNA is then electrophoresed to separate the mRNA species and the mRNA is transferred from the gel to a nitrocellulose membrane. As with the Southern blots, labeled probes are used to identify and/or quantify the *CYP24* mRNA.

b) Amplification-based assays.

[0087] In another preferred embodiment, *CYP24* transcript (e.g., *CYP24* mRNA) can be measured using amplification (e.g. PCR) based methods as described above for directly assessing copy number of *CYP24* DNA. In a preferred embodiment, *CYP24* transcript level is assessed by using reverse transcription PCR (RT-PCR). As mentioned above, because *CYP24* activity is tightly linked to vitamin D receptor (*VDR*) activity, where gene transcript level is being measured it is preferable to also measure *VDR* activity (e.g. transcript level). Then, an increase in *CYP24* activity for a given level of *VDR* activity indicates a cancer or an increased predisposition to cancer. Thus, in preferred amplification-based assays (e.g. RT-PCR) the level of *VDR* transcript is also assayed.

[0088] As indicated above, PCR assay methods are well known to those of skill in the art. Similarly, RT-PCR methods are also well known. Moreover, probes for such an RT-PCR assay are provided below in Table 1 and the assay is illustrated in Example 1 (see, e.g., Figure 3).

2) Detection of expressed protein

[0089] The "activity" of *CYP24* can also be detected and/or quantified by detecting or quantifying the expressed *CYP24* polypeptide. The polypeptide can be detected and quantified by any of a number of means well known to those of skill in the art. These may include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, or various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, western blotting, and the like.

[0090] In one preferred embodiment, the *CYP24* polypeptide is detected/quantified in an electrophoretic protein separation (e.g. a 1- or 2-dimensional electrophoresis). Means of detecting proteins using electrophoretic techniques are well known to those of skill in the art (see generally, R. Scopes (1982) Protein Purification, Springer-Verlag, N.Y.; Deutscher, (1990) Methods in Enzymology Vol. 182: Guide to Protein Purification, Academic Press, Inc., N.Y.).

[0091] In another preferred embodiment, Western blot (immunoblot) analysis is used to detect and quantify the presence of *CYP24* polypeptide in the sample. This technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind *CYP24* polypeptide. The anti-*CYP24* polypeptide antibodies specifically bind to *CYP24* on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (e.g., labeled sheep anti-mouse antibodies) that specifically bind to the anti-*CYP24*.

[0092] In a more preferred embodiment, the *CYP24* polypeptide is detected using an immunoassay. As used herein, an immunoassay is an assay that utilizes an antibody to specifically bind to the analyte (*CYP24* polypeptide). The immunoassay is thus characterized by detection of specific binding of a *CYP24* polypeptide to an anti-*CYP24* antibody as opposed to the use of other physical or chemical properties to isolate, target, and quantify the analyte.

[0093] The *CYP24* polypeptide is detected and/or quantified using any of a number of well recognized immunological binding assays (see, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also Asai (1993) Methods in Cell Biology Volume 37: Antibodies in Cell Biology. Academic Press, Inc. New York; Stites & Terr (1991) Basic and Clinical Immunology 7th Edition.

[0094] Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (in this case *CYP24* polypeptide or subsequence). The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds a *CYP24* polypeptide. The antibody (anti-*CYP24*) may be produced by any of a number of means well known to those of skill in the art.

[0095] Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled *CYP24* polypeptide or a labeled anti-*CYP24* antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/*CYP24* polypeptide complex.

[0096] In one preferred embodiment, the labeling agent is a second human *CYP24* antibody bearing a label. Alternatively, the second *CYP24* antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, e.g., as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

[0097] Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (see, generally Kronval, et al. (1973) J. Immunol., 111: 1401-1406, and Akerstrom (1985) J. Immunol., 135: 2589-2542).

[0098] As indicated above, immunoassays for the detection and/or quantification of *CYP24* polypeptide can take a wide variety of formats well known to those of skill in the art.

[0099] Preferred immunoassays for detecting *CYP24* polypeptide are either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte is directly measured. In one preferred "sandwich" assay, for example, the capture agent (anti-*CYP24* antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture *CYP24* polypeptide present in the test sample. The *CYP24* thus immobilized is then bound by a labeling agent, such as a second human *CYP24* antibody bearing a label.

[0100] In competitive assays, the amount of analyte (*CYP24* polypeptide) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte (*CYP24* polypeptide) displaced (or competed away) from a capture agent (anti *CYP24* antibody) by the analyte present in the sample. In one competitive assay, a known amount of, in this case, *CYP24* polypeptide is added to the sample and the sample is then contacted with a capture agent. The amount of *CYP24* polypeptide bound to the antibody is inversely proportional to the concentration of *CYP24* polypeptide

present in the sample.

[0101] In one particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of CYP24 polypeptide bound to the antibody may be determined either by measuring the amount of CYP24 polypeptide present in an CYP24 polypeptide/antibody complex, or alternatively by measuring the amount of remaining uncomplexed CYP24 polypeptide. The amount of CYP24 polypeptide may be detected by providing a labeled CYP24 polypeptide.

[0102] The assays of this invention are scored (as positive or negative or quantity of CYP24 polypeptide) according to standard methods well known to those of skill in the art. The particular method of scoring will depend on the assay format and choice of label. For example, a Western Blot assay can be scored by visualizing the colored product produced by the enzymatic label. A clearly visible colored band or spot at the correct molecular weight is scored as a positive result, while the absence of a clearly visible spot or band is scored as a negative. The intensity of the band or spot can provide a quantitative measure of CYP24.

[0103] Antibodies for use in the various immunoassays described herein, can be produced as described below.

3) Detection of enzyme activity.

[0104] In another embodiment, CYP24 level (activity) is assayed by measuring the enzymatic activity of the CYP24 polypeptide (25-hydroxyvitamin D3 24-hydroxylase enzyme). Methods of assaying the activity of this enzyme are well known to those of skill in the art. Thus, for example, CYP24 activity in cell suspensions will be assayed by measuring the metabolism of ³H-labeled 25OHD₃ (Amersham). The oxidation products are separated by HPLC and the activity calculated as the sum of the C-24 oxidation products (Tomon et al., 1990 Endocrinol., 126: 2868-2875). Alternatively, the CYP24 activity can be determined after incubation with 25-OH-[26,27-³H]D₃ (NEN #NET349) and measurement of radioactivity released as [³H]acetone after periodate cleavage (Beckman and DeLuca (1997) Meth. Enzymol., 282: 200-213).

C) Comparison of CYP24 levels while controlling for VDR activity.

[0105] As explained above, the activity level of CYP24 is tightly linked to the activity level of the vitamin D receptor (VDR). Thus, when assaying CYP24 activity (e.g. transcription, translation, activity of translated protein, etc.) the activity level is preferably determined with respect to the VDR activity level. When a sample tissue (e.g. tissue biopsy) shows a higher level of CYP24 activity than a control sample (e.g. healthy tissue) (preferably at the same level of VDR activity) then the elevated CYP24 activity indicates the presence of, prognosis of, or predisposition to develop, a cancer.

[0106] The VDR transcript (e.g., mRNA) levels or translated protein levels can be measured using the assays described above for CYP24 activity; the only difference being that the assay is adjusted for specificity to VDR nucleic acids or polypeptides rather than to CYP24.

[0107] Antibodies specific for VDR are commercially available (Affinity BioReagents #PA1-711, MA1-710, Santa Cruz Biotechnology # sc-1008, sc-1009). Gene specific probes for CYP24 and VDR mRNAs that can be used to generate riboprobes for mRNA FISH are provided in Example 1. In addition, an assay for CYP24 and VDR transcription levels is illustrated in Example 1.

D) Hybridization Formats and Optimization of hybridization conditions.

1) Array-based hybridization formats.

[0108] The methods of this invention are particularly well suited to array-based hybridization formats. For a description of one preferred array-based hybridization system see Pinkel et al. (1998) Nature Genetics, 20: 207-211.

[0109] Arrays are a multiplicity of different "probe" or "target" nucleic acids (or other compounds) attached to one or more surfaces (e.g., solid, membrane, or gel). In a preferred embodiment, the multiplicity of nucleic acids (or other moieties) is attached to a single contiguous surface or to a multiplicity of surfaces juxtaposed to each other.

[0110] In an array format a large number of different hybridization reactions can be run essentially "in parallel." This provides rapid, essentially simultaneous, evaluation of a number of hybridizations in a single "experiment". Methods of performing hybridization reactions in array based formats are well known to those of skill in the art (see, e.g., Pastinen (1997) Genome Res. 7: 606-614; Jackson (1996) Nature Biotechnology 14:1685; Chee (1995) Science 274: 610; WO 96/17958, Pinkel et al. (1998) Nature Genetics 20: 207-211).

[0111] Arrays, particularly nucleic acid arrays can be produced according to a wide variety of methods well known to those of skill in the art. For example, in a simple embodiment, "low density" arrays can simply be produced by spotting (e.g. by hand using a pipette) different nucleic acids at different locations on a solid support (e.g. a glass surface, a membrane, etc.).

[0112] This simple spotting, approach has been automated to produce high density spotted arrays (see, e.g., U.S.

Patent No: 5,807,522). This patent describes the use of an automated system that taps a microcapillary against a surface to deposit a small volume of a biological sample. The process is repeated to generate high density arrays.

[0113] Arrays can also be produced using oligonucleotide synthesis technology. Thus, for example, U.S. Patent No. 5,143,854 and PCT Patent Publication Nos. WO 90/15070 and 92/10092 teach the use of light-directed combinatorial synthesis of high density oligonucleotide arrays.

[0114] In brief, the light-directed combinatorial synthesis of oligonucleotide arrays on glass surfaces proceeds using automated phosphoramidite chemistry and chip masking techniques. In one specific implementation, a glass surface is derivatized with a silane reagent containing a functional group, e.g., a hydroxyl or amine group blocked by a photolabile protecting group. Photolysis through a photolithographic mask is used selectively to expose functional groups which are then ready to react with incoming 5'-photoprotected nucleoside phosphoramidites. The phosphoramidites react only with those sites which are illuminated (and thus exposed by removal of the photolabile blocking group). Thus, the phosphoramidites only add to those areas selectively exposed from the preceding step. These steps are repeated until the desired array of sequences have been synthesized on the solid surface. Combinatorial synthesis of different oligonucleotide analogues at different locations on the array is determined by the pattern of illumination during synthesis and the order of addition of coupling reagents.

[0115] In a preferred embodiment, the arrays used in this invention can comprise either probe or target nucleic acids. These probes or target nucleic acids are then hybridized respectively with their "target" nucleic acids. Because the *CYP24* gene sequence is known, oligonucleotide arrays can be synthesized containing one or multiple probes specific to *CYP24*.

[0116] In another embodiment the array, particularly a spotted array, can include genomic DNA, e.g. overlapping clones that provide a high resolution scan of the amplicon containing to *CYP24*, or of *CYP24* itself. Amplicon nucleic acid can be obtained from, e.g., HACs, MACs, YACs, BACs, PACs, P1s, cosmids, plasmids, inter-Alu PCR products of genomic clones, restriction digests of genomic clones, cDNA clones, amplification (e.g., PCR) products, and the like.

[0117] In various embodiments, the array nucleic acids are derived from previously mapped libraries of clones spanning or including the amplicon sequences of the invention, as well as clones from other areas of the genome, as described below. The arrays can be hybridized with a single population of sample nucleic acid or can be used with two differentially labeled collections (as with a test sample and a reference sample).

[0118] Many methods for immobilizing nucleic acids on a variety of solid surfaces are known in the art. A wide variety of organic and inorganic polymers, as well as other materials, both natural and synthetic, can be employed as the material for the solid surface. Illustrative solid surfaces include, e.g., nitrocellulose, nylon, glass, quartz, diazotized membranes (paper or nylon), silicones, polyformaldehyde, cellulose, and cellulose acetate. In addition, plastics such as polyethylene, polypropylene, polystyrene, and the like can be used. Other materials which may be employed include paper, ceramics, metals, metalloids, semiconductive materials, cermets or the like. In addition, substances that form gels can be used. Such materials include, e.g., proteins (e.g., gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

[0119] In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, proteins (e.g., bovine serum albumin) or mixtures of macromolecules (e.g., Denhardt's solution) can be employed to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like. If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature.

[0120] For example, methods for immobilizing nucleic acids by introduction of various functional groups to the molecules is known (see, e.g., Bischoff (1987) *Anal. Biochem.*, 164: 336-344; Kremsky (1987) *Nucl. Acids Res.* 15: 2891-2910). Modified nucleotides can be placed on the target using PCR primers containing the modified nucleotide, or by enzymatic end labeling with modified nucleotides. Use of glass or membrane supports (e.g., nitrocellulose, nylon, polypropylene) for the nucleic acid arrays of the invention is advantageous because of well developed technology employing manual and robotic methods of arraying targets at relatively high element densities. Such membranes are generally available and protocols and equipment for hybridization to membranes is well known.

[0121] Target elements of various sizes, ranging from 1 mm diameter down to 1 μm can be used. Smaller target elements containing low amounts of concentrated, fixed probe DNA are used for high complexity comparative hybridizations since the total amount of sample available for binding to each target element will be limited. Thus it is advantageous to have small array target elements that contain a small amount of concentrated probe DNA so that the signal that is obtained is highly localized and bright. Such small array target elements are typically used in arrays with densities greater than $10^4/\text{cm}^2$. Relatively simple approaches capable of quantitative fluorescent imaging of 1 cm^2 areas have been described that permit acquisition of data from a large number of target elements in a single image (see, e.g., Wittrup (1994) *Cytometry* 16:206-213, Pinkel et al. (1998) *Nature Genetics* 20: 207-211).

[0122] Arrays on solid surface substrates with much lower fluorescence than membranes, such as glass, quartz, or small beads, can achieve much better sensitivity. Substrates such as glass or fused silica are advantageous in that they provide a very low fluorescence substrate, and a highly efficient hybridization environment. Covalent attachment of the target nucleic acids to glass or synthetic fused silica can be accomplished according to a number of known techniques (described above). Nucleic acids can be conveniently coupled to glass using commercially available reagents. For instance, materials for preparation of silanized glass with a number of functional groups are commercially available or can be prepared using standard techniques (see, e.g., Gait (1984) *Oligonucleotide Synthesis: A Practical Approach*, IRL Press, Wash., D.C.). Quartz cover slips, which have at least 10-fold lower autofluorescence than glass, can also be silanized.

[0123] Alternatively, probes can also be immobilized on commercially available coated beads or other surfaces. For instance, biotin end-labeled nucleic acids can be bound to commercially available avidin-coated beads. Streptavidin or anti-digoxigenin antibody can also be attached to silanized glass slides by protein-mediated coupling using e.g., protein A following standard protocols (see, e.g., Smith (1992) *Science* 258: 1122-1126). Biotin or digoxigenin end-labeled nucleic acids can be prepared according to standard techniques. Hybridization to nucleic acids attached to beads is accomplished by suspending them in the hybridization mix, and then depositing them on the glass substrate for analysis after washing. Alternatively, paramagnetic particles, such as ferric oxide particles, with or without avidin coating, can be used.

[0124] In one particularly preferred embodiment, probe nucleic acid is spotted onto a surface (e.g., a glass or quartz surface). The nucleic acid is dissolved in a mixture of water, dimethylsulfoxide (DMSO), and nitrocellulose and spotted onto amino-silane coated glass slides. Small capillaries tubes can be used to "spot" the probe mixture.

2) Other hybridization formats.

[0125] A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in Hames and Higgins (1985) *Nucleic Acid Hybridization, A Practical Approach*, IRL Press; Gall and Pardue (1969) *Proc. Natl. Acad. Sci. USA* 63: 378-383; and John et al. (1969) *Nature* 223: 582-587.

[0126] Sandwich assays are commercially useful hybridization assays for detecting or isolating nucleic acid sequences. Such assays utilize a "capture" nucleic acid covalently immobilized to a solid support and a labeled "signal" nucleic acid in solution. The sample will provide the target nucleic acid. The "capture" nucleic acid and "signal" nucleic acid probe hybridize with the target nucleic acid to form a "sandwich" hybridization complex. To be most effective, the signal nucleic acid should not hybridize with the capture nucleic acid.

[0127] Typically, labeled signal nucleic acids are used to detect hybridization. Complementary nucleic acids or signal nucleic acids may be labeled by any one of several methods typically used to detect the presence of hybridized polynucleotides. The most common method of detection is the use of autoradiography with ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P -labelled probes or the like. Other labels include ligands that bind to labeled antibodies, fluorophores, chemi-luminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labeled ligand.

[0128] Detection of a hybridization complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such binding occurs through ligand and anti-ligand interactions as between a ligand-conjugated probe and an anti-ligand conjugated with a signal.

[0129] The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system that multiplies the target nucleic acid being detected. Examples of such systems include the polymerase chain reaction (PCR) system and the ligase chain reaction (LCR) system. Other methods recently described in the art are the nucleic acid sequence based amplification (NASBAO, Cangene, Mississauga, Ontario) and Q Beta Replicase systems.

3) Optimization of hybridization conditions.

[0130] Nucleic acid hybridization simply involves providing a denatured probe and target nucleic acid under conditions where the probe and its complementary target can form stable hybrid duplexes through complementary base pairing. The nucleic acids that do not form hybrid duplexes are then washed away leaving the hybridized nucleic acids to be detected, typically through detection of an attached detectable label. It is generally recognized that nucleic acids are denatured by increasing the temperature or decreasing the salt concentration of the buffer containing the nucleic acids, or in the addition of chemical agents, or the raising of the pH. Under low stringency conditions (e.g., low temperature and/or high salt and/or high target concentration) hybrid duplexes (e.g., DNA:DNA, RNA:RNA, or RNA:DNA) will form even where the annealed sequences are not perfectly complementary. Thus specificity of hybridization is reduced at lower stringency. Conversely, at higher stringency (e.g., higher temperature or lower salt) successful hybridization requires fewer mismatches.

[0131] One of skill in the art will appreciate that hybridization conditions may be selected to provide any degree of

stringency. In a preferred embodiment, hybridization is performed at low stringency to ensure hybridization and then subsequent washes are performed at higher stringency to eliminate mismatched hybrid duplexes. Successive washes may be performed at increasingly higher stringency (e.g., down to as low as 0.25 X SSPE at 37°C to 70°C) until a desired level of hybridization specificity is obtained. Stringency can also be increased by addition of agents such as formamide. Hybridization specificity may be evaluated by comparison of hybridization to the test probes with hybridization to the various controls that can be present.

[0132] In general, there is a tradeoff between hybridization specificity (stringency) and signal intensity. Thus, in a preferred embodiment, the wash is performed at the highest stringency that produces consistent results and that provides a signal intensity greater than approximately 10% of the background intensity. Thus, in a preferred embodiment, the hybridized array may be washed at successively higher stringency solutions and read between each wash. Analysis of the data sets thus produced will reveal a wash stringency above which the hybridization pattern is not appreciably altered and which provides adequate signal for the particular probes of interest.

[0133] In a preferred embodiment, background signal is reduced by the use of a detergent (e.g., C-TAB) or a blocking reagent (e.g., tRNA, sperm DNA, cot-1 DNA, etc.) during the hybridization to reduce non-specific binding. In a particularly preferred embodiment, the hybridization is performed in the presence of about 10 µg/l µL tRNA. The use of blocking agents in hybridization is well known to those of skill in the art (see, e.g., Chapter 8 in P. Tijssen, *supra*.)

[0134] Methods of optimizing hybridization conditions are well known to those of skill in the art (see, e.g., Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 24: Hybridization With Nucleic Acid Probes, Elsevier, N.Y.).

[0135] Optimal conditions are also a function of the sensitivity of label (e.g., fluorescence) detection for different combinations of substrate type, fluorochrome, excitation and emission bands, spot size and the like. Low fluorescence background surfaces can be used (see, e.g., Chu (1992) *Electrophoresis* 13:105-114). The sensitivity for detection of spots ("target elements") of various diameters on the candidate surfaces can be readily determined by, e.g., spotting a dilution series of fluorescently end labeled DNA fragments. These spots are then imaged using conventional fluorescence microscopy. The sensitivity, linearity, and dynamic range achievable from the various combinations of fluorochrome and solid surfaces (e.g., glass, fused silica, etc.) can thus be determined. Serial dilutions of pairs of fluorochrome in known relative proportions can also be analyzed. This determines the accuracy with which fluorescence ratio measurements reflect actual fluorochrome ratios over the dynamic range permitted by the detectors and fluorescence of the substrate upon which the probe has been fixed.

4) Labeling and detection of nucleic acids.

[0136] In a preferred embodiment, the hybridized nucleic acids are detected by detecting one or more labels attached to the sample nucleic acids. The labels may be incorporated by any of a number of means well known to those of skill in the art. Means of attaching labels to nucleic acids include, for example nick translation, or end-labeling by kinasing of the nucleic acid and subsequent attachment (ligation) of a linker joining the sample nucleic acid to a label (e.g., a fluorophore). A wide variety of linkers for the attachment of labels to nucleic acids are also known. In addition, intercalating dyes and fluorescent nucleotides can also be used.

[0137] Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads™), fluorescent dyes (e.g., fluorescein, texas red, rhodamine, green fluorescent protein, and the like, see, e.g., *Molecular Probes*, Eugene, Oregon, USA), radiolabels (e.g., ³H, ¹²⁵I, ³⁵S, ¹⁴C, or ³²P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold (e.g., gold particles in the 40 -80 nm diameter size range scatter green light with high efficiency) or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

[0138] A fluorescent label is preferred because it provides a very strong signal with low background. It is also optically detectable at high resolution and sensitivity through a quick scanning procedure. The nucleic acid samples can all be labeled with a single label, e.g., a single fluorescent label. Alternatively, in another embodiment, different nucleic acid samples can be simultaneously hybridized where each nucleic acid sample has a different label. For instance, one target could have a green fluorescent label and a second target could have a red fluorescent label. The scanning step will distinguish sites of binding of the red label from those binding the green fluorescent label. Each nucleic acid sample (target nucleic acid) can be analyzed independently from one another.

[0139] Suitable chromogens which can be employed include those molecules and compounds which absorb light in a distinctive range of wavelengths so that a color can be observed or, alternatively, which emit light when irradiated with radiation of a particular wave length or wave length range, e.g., fluorescers.

[0140] Desirably, fluorescers should absorb light above about 300 nm, preferably about 350 nm, and more preferably

above about 400 nm, usually emitting at wavelengths greater than about 10 nm higher than the wavelength of the light absorbed. It should be noted that the absorption and emission characteristics of the bound dye can differ from the unbound dye. Therefore, when referring to the various wavelength ranges and characteristics of the dyes, it is intended to indicate the dyes as employed and not the dye which is unconjugated and characterized in an arbitrary solvent.

5 [0141] Fluorosceners are generally preferred because by irradiating a fluoescer with light, one can obtain a plurality of emissions. Thus, a single label can provide for a plurality of measurable events.

[0142] Detectable signal can also be provided by chemiluminescent and bioluminescent sources. Chemiluminescent sources include a compound which becomes electronically excited by a chemical reaction and can then emit light which serves as the detectable signal or donates energy to a fluorescent acceptor. Alternatively, luciferins can be used in
10 conjunction with luciferase or lucigenins to provide bioluminescence.

[0143] Spin labels are provided by reporter molecules with an unpaired electron spin which can be detected by electron spin resonance (ESR) spectroscopy. Exemplary spin labels include organic free radicals, transitional metal complexes, particularly vanadium, copper, iron, and manganese, and the like. Exemplary spin labels include nitroxide free radicals.

15 [0144] The label may be added to the target (sample) nucleic acid(s) prior to, or after the hybridization. So called "direct labels" are detectable labels that are directly attached to or incorporated into the target (sample) nucleic acid prior to hybridization. In contrast, so called "indirect labels" are joined to the hybrid duplex after hybridization. Often, the indirect label is attached to a binding moiety that has been attached to the target nucleic acid prior to the hybridization. Thus, for example, the target nucleic acid may be biotinylated before the hybridization. After hybridization, an avidin-conjugated fluorophore will bind the biotin bearing hybrid duplexes providing a label that is easily detected. For a detailed review of methods of labeling nucleic acids and detecting labeled hybridized nucleic acids see Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 24: Hybridization With Nucleic Acid Probes, P. Tijssen, ed. Elsevier, N.Y., (1993)).

[0145] Fluorescent labels are easily added during an *in vitro* transcription reaction. Thus, for example, fluorescein labeled UTP and CTP can be incorporated into the RNA produced in an *in vitro* transcription.

25 [0146] The labels can be attached directly or through a linker moiety. In general, the site of label or linker-label attachment is not limited to any specific position. For example, a label may be attached to a nucleoside, nucleotide, or analogue thereof at any position that does not interfere with detection or hybridization as desired. For example, certain Label-ON Reagents from Clontech (Palo Alto, CA) provide for labeling interspersed throughout the phosphate backbone of an oligonucleotide and for terminal labeling at the 3' and 5' ends. As shown for example herein, labels can be attached
30 at positions on the ribose ring or the ribose can be modified and even eliminated as desired. The base moieties of useful labeling reagents can include those that are naturally occurring or modified in a manner that does not interfere with the purpose to which they are put. Modified bases include but are not limited to 7-deaza A and G, 7-deaza-8-aza A and G, and other heterocyclic moieties.

35 [0147] It will be recognized that fluorescent labels are not to be limited to single species organic molecules, but include inorganic molecules, multi-molecular mixtures of organic and/or inorganic molecules, crystals, heteropolymers, and the like. Thus, for example, CdSe-CdS core-shell nanocrystals enclosed in a silica shell can be easily derivatized for coupling to a biological molecule (Bruchez et al. (1998) Science, 281: 2013-2016). Similarly, highly fluorescent quantum dots (zinc sulfide-capped cadmium selenide) have been covalently coupled to biomolecules for use in ultrasensitive biological detection (Warren and Nie (1998) Science, 281: 2016-2018).

40 E) Antibodies to CYP24.

[0148] Either polyclonal or monoclonal antibodies may be used in the immunoassays and therapeutic methods of the invention described herein. Polyclonal antibodies are preferably raised by multiple injections (*e.g.* subcutaneous or
45 intramuscular injections) of substantially pure CYP24 polypeptides or antigenic CYP24 polypeptides into a suitable non-human mammal. The antigenicity of CYP24 peptides can be determined by conventional techniques to determine the magnitude of the antibody response of an animal that has been immunized with the peptide. Generally, the CYP24 peptides that are used to raise the anti-CYP24 antibodies should generally be those which induce production of high titers of antibody with relatively high affinity for CYP24.

50 [0149] If desired, the immunizing peptide may be coupled to a carrier protein by conjugation using techniques which are well-known in the art. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (*e.g.* a mouse or a rabbit). Because CYP24 may be conserved among mammalian species, use of a carrier protein to enhance the immunogenicity of CYP24 proteins is preferred.

55 [0150] The antibodies are then obtained from blood samples taken from the mammal. The techniques used to develop polyclonal antibodies are known in the art (see, *e.g.*, Methods of Enzymology, "Production of Antisera With Small Doses of Immunogen: Multiple Intradermal Injections", Langone, et al. eds. (Acad. Press, 1981)). Polyclonal antibodies produced by the animals can be further purified, for example, by binding to and elution from a matrix to which the peptide to which

the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies see, for example, Coligan, et al. (1991) Unit 9, Current Protocols in Immunology, Wiley Interscience).

5 **[0151]** Preferably, however, the *CYP24* antibodies produced will be monoclonal antibodies ("mAb's"). For preparation of monoclonal antibodies, immunization of a mouse or rat is preferred. The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as, Fab and F(ab')₂' which are capable of binding an epitopic determinant. Also, in this context, the term "mab's of the invention" refers to monoclonal antibodies with specificity for *CYP24*.

10 **[0152]** The general method used for production of hybridomas secreting mAbs is well known (Kohler and Milstein (1975) Nature, 256:495). Briefly, as described by Kohler and Milstein the technique comprised isolating lymphocytes from regional draining lymph nodes of five separate cancer patients with either melanoma, teratocarcinoma or cancer of the cervix, glioma or lung, (where samples were obtained from surgical specimens), pooling the cells, and fusing the cells with SHFP-1. Hybridomas were screened for production of antibody which bound to cancer cell lines.

15 **[0153]** Confirmation of *CYP24* specificity among mAb's can be accomplished using relatively routine screening techniques (such as the enzyme-linked immunosorbent assay, or "ELISA") to determine the elementary reaction pattern of the mAb of interest.

20 **[0154]** It is also possible to evaluate an mAb to determine whether it has the same specificity as a mAb of the invention without undue experimentation by determining whether the mAb being tested prevents a mAb of the invention from binding to *CYP24* isolated as described above. If the mAb being tested competes with the mAb of the invention, as shown by a decrease in binding by the mAb of the invention, then it is likely that the two monoclonal antibodies bind to the same or a closely related epitope. Still another way to determine whether a mAb has the specificity of a mAb of the invention is to preincubate the mAb of the invention with an antigen with which it is normally reactive, and determine if the mAb being tested is inhibited in its ability to bind the antigen. If the mAb being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the mAb of the invention.

25 **[0155]** Antibodies fragments, e.g. single chain antibodies (scFv or others), can also be produced/selected using phage display technology. The ability to express antibody fragments on the surface of viruses that infect bacteria (bacteriophage or phage) makes it possible to isolate a single binding antibody fragment from a library of greater than 10¹⁰ nonbinding clones. To express antibody fragments on the surface of phage (phage display), an antibody fragment gene is inserted into the gene encoding a phage surface protein (pIII) and the antibody fragment-pIII fusion protein is displayed on the phage surface (McCafferty et al. (1990) Nature, 348: 552-554; Hoogenboom et al. (1991) Nucleic Acids Res. 19: 4133-4137).

30 **[0156]** Since the antibody fragments on the surface of the phage are functional, phage bearing antigen binding antibody fragments can be separated from non-binding phage by antigen affinity chromatography (McCafferty et al. (1990) Nature, 348: 552-554). Depending on the affinity of the antibody fragment, enrichment factors of 20 fold -1,000,000 fold are obtained for a single round of affinity selection. By infecting bacteria with the eluted phage, however, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000 fold in one round can become 1,000,000 fold in two rounds of selection (McCafferty et al. (1990) Nature, 348: 552-554). Thus even when enrichments are low (Marks et al. (1991) J. Mol. Biol. 222: 581-597), multiple rounds of affinity selection can lead to the isolation of rare phage. Since selection of the phage antibody library on antigen results in enrichment, the majority of clones bind antigen after as few as three to four rounds of selection. Thus only a relatively small number of clones (several hundred) need to be analyzed for binding to antigen.

35 **[0157]** Human antibodies can be produced without prior immunization by displaying very large and diverse V-gene repertoires on phage (Marks et al. (1991) J. Mol. Biol. 222: 581-597). In one embodiment natural V_H and V_L repertoires present in human peripheral blood lymphocytes are were isolated from unimmunized donors by PCR. The V-gene repertoires were spliced together at random using PCR to create a scFv gene repertoire which is was cloned into a phage vector to create a library of 30 million phage antibodies (*Id.*). From this single "naive" phage antibody library, binding antibody fragments have been isolated against more than 17 different antigens, including haptens, polysaccharides and proteins (Marks et al. (1991) J. Mol. Biol. 222: 581-597; Marks et al. (1993). Bio/Technology. 10: 779-783; Griffiths et al. (1993) EMBO J. 12: 725-734; Clackson et al. (1991) Nature. 352: 624-628). Antibodies have been produced against self proteins, including human thyroglobulin, immunoglobulin, tumor necrosis factor and CEA (Griffiths et al. (1993) EMBO J. 12: 725-734). It is also possible to isolate antibodies against cell surface antigens by selecting directly on intact cells. The antibody fragments are highly specific for the antigen used for selection and have affinities in the 1 μM to 100 nM range (Marks et al. (1991) J. Mol. Biol. 222: 581-597; Griffiths et al. (1993) EMBO J. 12: 725-734). Larger phage antibody libraries result in the isolation of more antibodies of higher binding affinity to a greater proportion of antigens.

55 **[0158]** It will also be recognized that *CYP24* antibodies can be prepared by any of a number of commercial services (e.g., Berkeley antibody laboratories, Bethyl Laboratories, Anawa, Eurogenetec, etc.).

II. Assay Optimization- Determining prognostically significant levels.

[0159] The assays of this invention have immediate utility in detecting/predicting the likelihood of a cancer, in estimating survival from a cancer, in screening for agents that modulate *CYP24* activity, and in screening for agents that inhibit cell proliferation. In particular, for example, identification of an amplification in *CYP24* (genomic DNA) indicates the presence of a cancer and/or the predisposition to develop a cancer.

[0160] Methods of optimizing predictive/diagnostic assays are well known to those of ordinary skill in the art. Typically this involves determining "baseline levels" (e.g. of *CYP24*) in normal tissues and *CYP24* activity levels in pathological (i.e. tumor tissues). In particularly preferred embodiments, such levels are determined with appropriate controls for concurrent *VDR* activity, sample type, age, sex, developmental state, overall physiological state (e.g. in a non-pregnant as compared to a pregnant female), overall health, tumor type, etc. In a preferred embodiment, "baseline" (e.g., control) levels are determined from a normal (healthy) tissue from the same individual or from individuals of the same population. Alternatively, "baseline" and "pathological" levels are determined from "population" studies that provide sufficient sample size and diversity that the influence of the various cofactors identified above (age, health, sex, etc.) can be of statistically evaluated. "Baseline" *CYP24* levels can also be evaluated by reference to model systems, e.g., as described in Examples 3-5.

[0161] In a preferred embodiment, quantitative assays of *CYP24* level are deemed to show a positive result, e.g. elevated *CYP24* level, when the measured *CYP24* level is greater than the level measured or known for a control sample (e.g. either a level known or measured for a normal healthy mammal of the same species or a "baseline/reference" level determined at a different tissue and/or a different time for the same individual. In a particularly preferred embodiment, the assay is deemed to show a positive result (e.g., "a prognostically significant level") when the difference between sample and "control" is statistically significant (e.g. at the 85% or greater, preferably at the 90% or greater, more preferably at the 95% or greater and most preferably at the 98% or greater confidence level).

III. Methods of treating cancer -- selection of adjuvant therapy based on *CYP24* level.

[0162] Because of the ability to evaluate the presence of, or the predisposition to develop, a cancer, the assays of this invention make a useful component of a cancer therapy regimen. Thus, in one embodiment, *CYP24* activity can be used as a measure of disease progression, while in another embodiment *CYP24* activity is used to evaluate the necessity of an adjuvant therapy.

[0163] "Adjuvant cancer therapy" refers to a method of treating cancer, such as chemotherapy, radiation therapy, surgery, reoperation, antihormone therapy, and immunotherapy, that is administered in combination with or following another method of cancer treatment. An "adjuvant cancer therapy" often represents an aggressive form of cancer treatment that is selected in view of a reduced survival expectancy and/or a detected level of *CYP24* that is elevated compared to a control level.

Adjuvant therapies are well known to those of skill in the art and include, but are not limited to chemotherapy, radiation therapy, primary surgery or reoperation, antihormone therapy, immunotherapy, and the like. "Chemotherapy", as used in this context, refers to the administration of chemical compounds to an animal with cancer that is aimed at killing or reducing the number of cancer cells within the animal. Generally, chemotherapeutic agents arrest the growth of or kill cells that are dividing or growing, such as cancer cells. Chemotherapeutic agents for use against cancer are well known to those of skill in the art include, but are not limited to doxorubicin, vinblastine, genistein, etc.

[0164] "Radiation therapy" in this context refers to the administration of radioactivity to an animal with cancer. Radiation kills or inhibits the growth of dividing cells, such as cancer cells. The administration may be by an external source (e.g., a gamma source, a proton source, a molecular beam source, etc.) or may be by an implantable radioactive material. Radiation therapy includes "traditional" radiation treatment aimed at reduction or elimination of tumor volume or more aggressive radio-surgery techniques.

[0165] Surgical methods refer to the direct removal or ablation of cells, e.g. cancer cells, from an animal. Most often, the cancer cells will be in the form of a tumor (e.g. a mammary tumor), which is removed from the animal. The surgical methods may involve removal of healthy as well as pathological tissue. "Reoperation" refers to surgery performed on an animal that has previously undergone surgery for treatment of the same pathology.

[0166] "Antihormone therapy" refers to the administration of compounds that counteract or inhibit hormones, such as estrogen or androgen, that have a mitogenic effect on cells. Often, these hormones act to increase the cancerous properties of cancer cells *in vivo*.

[0167] Immunotherapy refers to methods of enhancing the ability of an animal's immune system to destroy cancer cells within the animal. This can involve the treatment with polyclonal or monoclonal antibodies that bind particular tumor-specific markers (e.g. IL-13 receptor, and Lewis Y (Le^Y) marker, etc.) help to direct cytotoxins of native immune system effectors to the tumor target. Immunotherapeutic methods are well known to those of skill in the art (see, e.g. Pastan et al. (1992) Ann. Rev. Biochem., 61: 331-354, Brinkman and Pastan (1994) Biochimica Biophysica Acta, 1198:27-45, etc.).

IV. Kits for use in diagnostic and/or prognostic applications.

[0168] For use in diagnostic and research, suggested above, kits are also described herein. In the diagnostic and research applications such kits may include any or all of the following: assay reagents, buffers, *CYP24* specific and/or VDR specific nucleic acids or antibodies (e.g. full-size monoclonal or polyclonal antibodies, single chain antibodies (e.g., scPv), or other *CYP24* or *VDR* binding molecules), and other hybridization probes and/or primers, and/or substrates for 25-hydroxyvitamin D3 24-hydroxylase.

[0169] In addition, the kits may include instructional materials containing directions (i.e., protocols) for the practice of the methods of this invention. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

[0170] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: Identification of *CYP24* as a driver Oncogene for Amplification at 20q13.2.

[0171] This experiment describes genetic analysis of breast tumors that indicates selective amplification of *CYP24*. Selection for higher copy number of this gene during tumor evolution provides further evidence of the importance of the vitamin D pathway in tumor development in the breast.

[0172] We have used a new high resolution form of comparative genomic hybridization, array CGH, to obtain a high resolution, quantitative map of DNA copy number across a region of recurrent amplification at chromosome band 20q13.2. Array CGH, which was developed in our laboratories uses microarrays of DNA clones as the hybridization target so that its resolution is determined by the spacing of the target clones across a genomic region (Figure 1). Thus, when contiguous clones make up the array, very high resolution copy number profiles can be obtained.

[0173] The unprecedented high dynamic range and quantitative accuracy of array CGH provides for the first time, the capability to very precisely map copy number profiles across an amplified region. In some tumors, copy number profiles show narrow peaks of amplification (~300 kb in Figure 3). This information focuses attention on genes mapping to the region and indicates that they should be given highest priority for evaluation as candidate driver oncogenes. The application of high resolution array CGH across region A at chromosome band 20q13.2 in breast cancer revealed the existence of two subregions, A1 and A2 with distinct amplification behavior. Recently a candidate oncogene, *ZNF217* (Collins *et al.*, 1998) has been identified that maps to subregion A1 and is likely to be the driver gene for amplification of A1. Our attention is now focused on the gene *CYP24* as the driver oncogene for region A2, because it maps to the narrow genomic interval most highly amplified in these tumors.

[0174] Previously, *CYP24* had been discounted as a candidate oncogene because it was not found to be transcribed in the breast cancer cell line, BT474 (Collins *et al.* (1998) Proc. Natl. Acad. Sci. USA 95: 8703-8708). However, re-evaluation of expression of *CYP24* in cell lines and tumors was warranted because of its position at the peak of the copy number profile and because of the existing knowledge of its function. Therefore we examined expression levels of *CYP24* and the vitamin D receptor (*VDR*), which controls *CYP24* expression by RT-PCR using the primers listed in Table 1. This re-evaluation shows that these genes are expressed in breast cancer cell lines and tumors (Figure 3). Expression of *CYP24* and *VDR* was detected in MCF7 cells and higher levels of expression of *CYP24* were induced when cells were treated with 1,25-dihydroxyvitamin-D3 (Figure 3A). Furthermore, expression of *CYP24* and *VDR* was detected in two breast tumors S21 and S59 (Figure 3B). In BT474 however, *CYP24* expression was not detected without addition of 1,25-dihydroxyvitamin-D3 to the culture medium (Figure 3C). Only low level expression of *VDR* was found in this cell line, most likely accounting for the failure to detect expression of *CYP24* in BT474 without addition of 1,25-dihydroxyvitamin-D3. These observations on BT474 illustrate the complexity of the analysis of *CYP24* function and emphasize the importance of measuring *VDR* activity when evaluating the role of *CYP24* in tumorigenesis.

Table 1. Primers used for assessing gene expression of *CYP24* and *VDR*.

Primer Name	Sequence	SEQ ID NO
<i>CYP24</i> Forward	5' - (AAT TAA CCC TCA CTA AAG GG) CAA ACC GTG GAA GGC CTA TC -3' *	1

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(continued)

Primer Name	Sequence	SEQ ID NO
CYP24 Reverse	5' - (TAA TAC GAC TCA CTA TAG GGA G) T CTT CCC TTC CAG GAT CA-3' **	2
VDR.Forward	5'-CTTCAGGCGAAGCATGAAGC-3' +	3
VDR Reverse	5'-CCTTCATCATGCCGATGTCC-3'	4
ZNF217 Forward	5' - (AAT TAA CCC TCA CTA AAG GG) AGA GGG GTG AGT GAC AAG-3' *	5
ZNF217 Reverse	5' - (TAA TAC GAC TCA CTA TAG GG) AGC TCG GAA TGG AAC AAC-3' ^a	6

*T3 promoter shown in parentheses is included at the 5' end so that the amplified product can be used as a template for *in vitro* transcription to generate riboprobes for mRNA FISH.
 **T7 promoter shown in parentheses is included at the 5' end so that the amplified product can be used as a template for *in vitro* transcription to generate riboprobes for mRNA FISH. The reverse primer spans the second exon-exon junction, preventing amplification of genomic DNA. A 111 bp fragment is amplified.
 +Spans the third exon-exon junction.
 ++ A 134 bp PCR fragment is amplified.
^aA 265 bp PCR fragment is amplified.

Example 2: Expression analysis Using Multi-color Fluorescent *in situ* Hybridization (mRNA FISH) on Tissue Sections.

[0175] In order to identify genes that are overexpressed in tumor compared to normal tissue, we have adapted our FISH protocols for visualizing transcription patterns in *C. elegans* (Albertson et al. (1995) pages 339-364 In *C. elegans: Modern Biological Analysis of an Organism*, vol. 48, H. F. Epstein and D. C. Shakes, eds. Academic Press, Inc; Birchall et al. (1995) *Nature Genet.* 11: 314-320) for use with formalin fixed paraffin embedded clinical specimens. Our approach involves the use of fluorescently labeled riboprobes that are synthesized by *in vitro* transcription. The DNA template for the transcription reaction is generated by amplification using gene specific primers in which the T3 or T7 phage promoter has been incorporated in the 5' end. Thus, subcloning of gene fragments to make probes can be avoided.

[0176] The hybridization signal was imaged with a confocal microscope, that reduces interference from tissue autofluorescence because of the narrow wavelength exciting light and the exclusion of out of focus fluorescence. The use of fluorescent probes, rather than radioactive probes has a number of advantages including, higher resolution, time saving, compatibility with simultaneous immunohistochemistry (Chuang et al. (1996) *Cell*, 79: 1-20) and the possibility of measuring relative levels of expression of a number of genes simultaneously on a single tissue section (Albertson *et al.*, 1995).

Example 3: Expression of CYP24 and VDR in normal mammary cells

[0177] In the human, vitamin D receptors have been localized by immunohistochemistry to the luminal and alveolar epithelial cells of the normal breast and in breast tumor cells (Berger et al. (1987) *Cancer Res.* 47: 6793-6799; Colston et al. (1989) *Lancet*, 188-191). In this experiment, the expression profiles of the *CYP24* and *VDR* genes during various stages of murine mammary gland development and involution are determined in order to identify the cell types and developmental stages in which these gene products function. These studies will provide the description of the normal expression of these genes, which are then compared to expression in murine breast tumor models and the *CYP24* transgenic mouse to be developed as described below.

[0178] The expression analysis is carried out at both the transcript and protein levels. As described above gene specific probes for *CYP24* and *VDR* mRNAs can be used to generate riboprobes for mRNA FISH. Antibodies specific for *VDR* are commercially available (Affinity BioReagents #PA1-711, MA1-710; Santa Cruz Biotechnology #sc-1008, sc-1009)

[0179] Immunohistochemistry and/or a combination of mRNA FISH and immunohistochemistry are used to localize the site of expression of particular genes and marker proteins specific for various cell types in the breast. Where possible, localization of expression of *CYP24* and/or *VDR* and the cell type specific markers is carried out simultaneously on the

same tissue sections using multiple distinguishable fluorochromes on the probes for the genes and marker proteins.

[0180] Development of the rodent breast has been described (see, e.g., Medina (1996) J. Mamm. Gland Biol. Neopl. 1: 5-19) and begins by arborization of the ductal system throughout the mammary fat pad at 4-8 weeks of age. The terminal end-buds, located at the leading edge of the invading ducts contain proliferating cells. At the time of pregnancy, further arborization of the ductal system takes place by elaboration of tertiary end-buds from the sides of the existing ductal tree. Terminal differentiation of the gland takes place during lactation when the milk proteins, lactoglobulin and whey acidic protein are synthesized. Involution of the lactating mammary gland involves extensive apoptosis and occurs during 4-6 weeks following weaning.

Experimental Design.

[0181] Expression of *CYP24* and *VDR* is determined by *in situ* staining of tissue sections using mRNA FISH or gene specific antibodies. Tissue blocks are prepared following *in vivo* perfusion and fixation of the mice. Mammary glands are harvested from mice at: (a) the beginning and end of breast ductal arborization (at 3-4 weeks and at 8 weeks, respectively), (b) at early, intermediate and late stages of pregnancy (at 4, 8, 13, and 18 days post coitus), (c) during lactation, and (d) during early and late breast involution (at 4 and 8 weeks after elective weaning). Prior to sacrifice, all mice are injected with 5-bromodeoxyuridine (BrdU) for immunohistochemical detection of S-phase cells using monoclonal BrdU antibody (Arbeit, *et al.*, 1994). Immunohistochemical staining of keratin intermediate filament proteins is used to distinguish the basal (keratin-14) and luminal cells (keratin-6) of the ducts (Antibodies, BabCo # prb-155p, -169p). The early and late stages of involution is identified by using the TUNEL assay for apoptosis (Naik *et al.* (1996) Genes Dev. 10: 2106-2166).

Methods

Specimen preparation.

[0182] Mice are injected i.p. with 100 mg/kg BrdU 2 hrs prior to sacrifice. The mice are weighed and anestheized with 37.5 mg/kg of a 0.25% Avertin solution and perfused with a 3.75% solution of freshly prepared paraformaldehyde. Tissues are removed, post-fixed overnight in 3.75% paraformaldehyde at 4°C and then embedded in paraffin. Sections (~6µm thickness) are de-waxed in xylene, taken through a graded series of ethanols and then incubated with 5-15 µg/ml of proteinase K at 37°C for 15 min., depending on the application. Following protease treatment the specimens are post-fixed in 1% paraformaldehyde for 20 min. at room temperature, rinsed and then dehydrated.

mRNA FISH.

[0183] Specimens are pre-hybridized in hybridization buffer (50-70% formamide, 5 X SSC, 0.1% SDS, 0.1% Tween 20, 100µg/ml tRNA, 10% dextran sulfate) at 37°C for 2 hrs. The pre-hybridization solution is removed and the fluorescently labeled riboprobe (Albertson *et al.* (1995) pages 339-364 In *C. elegans: Modern Biological Analysis of an Organism*, vol. 48, H. F. Epstein and D. C. Shakes, eds. Academic Press, Inc) are applied to the specimen in hybridization buffer. Hybridization is carried out overnight at 37-50°C depending on the length and GC content of the probe.

Immunohistochemistry.

[0184] Processing of sections varies slightly depending on the antibody, but will use standard methods for indirect detection (e.g. Albertson (1984) Develop. Biol. 101: 61-72).

S phase analysis.

[0185] After immunohistochemical staining for BrdU positive cells, the BrdU labeling index is determined by counting 1000 nuclei in sequential 20X fields.

Apoptosis.

[0186] The TUNEL assay is carried out using fluorescent detection of terminal transferase activity according to the manufacturer's directions (Oncor # S7110, Gaiterburg, MD).

Data Collection and Analysis.

[0187] Expression profiles of *CYP24* and *VDR* in mammary tissue includes enumeration of specific cell types, developmental stage-specific expression patterns and relative levels of expression. Expression of cell specific marker proteins is used to confirm assignment of *CYP24* and *VDR* expression in particular cells and developmental stages. The basal and luminal cells of the ducts are distinguished by their expression of particular keratins and proliferating terminal end-buds will be identified by BrdU incorporation. Involuting cells are identified as TUNEL positive cells. The relative levels of expression of *CYP24* and *VDR* mRNA at the different developmental stages are measured relative to a ribosomal probe hybridized to the same tissue sections.

Example 4: Expression of *CYP24* in the Established Murine Breast Cancer Model, MMTV-ERBB2 transgenic mouse.

[0188] We have documented the expression of *CYP24* and *VDR* in human breast tumor specimens (Figure 3) and will continue to survey expression of these genes in normal and tumor tissue from human breast tumor specimens. Here, we will investigate the expression of *CYP24* and *VDR* during breast carcinogenesis in an established transgenic mouse model of breast cancer, in which the *ERBB2* oncogene is expressed in mammary tissue under the control of the mouse mammary tumor virus promoter (JAX Mice, MMTVneu Erbb2, #002376). These mice first develop focal tumors in hyperplastic, dysplastic mammary glands at ~4 months (Guy *et al.*, 1992). The study of transgenic mouse models of breast carcinogenesis, offers the opportunity to investigate the potential role of these genes in certain aspects of tumorigenesis that cannot be studied by analysis of patient material. In particular, mouse models offer access to premalignant stages generally not available from human specimens. Furthermore, murine tumor models allow the role of particular genes in tumorigenesis to be assessed in tumors induced in a defined genetic background (e.g. tumors induced by overexpression of *ERBB2*, cyclin D1 or loss of p53).

Experimental Design.

[0189] Transgenic mice of 2, 4, 6 and 10-12 months are studied to encompass time points of early and late tumor development. Two hours prior to sacrifice, BrdU is injected intra-peritoneally to measure S-phase kinetics. Tissues are harvested and processed for mRNA FISH, and expression of keratins-14 and -6, and the *HER2-neu* transgene are determined, using antibodies as in Example 3.

[0190] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

Claims

1. A method of detecting a predisposition to cancer in an animal said method comprising:

- (i) using a biological sample from said animal;
- (ii) detecting the level of *CYP24* within said biological sample; and
- (iii) comparing said level of *CYP24* with a level of *CYP24* in a control sample taken from a normal, cancer-free tissue;

wherein an increased level of *CYP24* in said biological sample compared to the level of *CYP24* in said control sample indicates a predisposition to cancer in said animal.

2. The method of claim 1, wherein said level of *CYP24* is detected by determining the copy number of *CYP24* genes in the cells of said biological sample.
3. The method of claim 2, wherein said copy number is measured using Comparative Genomic Hybridization (CGH).
4. The method of claim 2, wherein said copy number is determined by hybridization to an array of nucleic acid probes.
5. The method of claim 3, wherein said Comparative Genomic Hybridization is performed on an array.

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6. The method of claim 1, wherein said level of *CYP24* is detected by measuring the level of *CYP24* mRNA in said biological sample, wherein an increased level of *CYP24* RNA in said sample compared to *CYP24* RNA in said control sample indicates a predisposition to cancer.
- 5 7. The method of claim 6, wherein said level of *CYP24* mRNA is measured in said biological sample and said control sample at the same vitamin D receptor activity or the *CYP24* mRNA levels are normalized to the level of vitamin D receptor activity in the sample and control.
8. The method of claim 6, wherein said level of *CYP24* mRNA is measured by hybridization to one or more probes on
10 an array.
9. The method of claim 1, wherein said level of *CYP24* is detected by measuring the level of *CYP24* protein in said biological sample, wherein an increased level of *CYP24* protein in said sample as compared to *CYP24* protein in said control sample indicates a predisposition to cancer.
- 15 10. The method of claim 9, wherein the level of *CYP24* protein is measured in the biological sample and the control sample at the same vitamin D receptor activity or the protein levels are normalized to the level of vitamin D receptor activity in the sample and control.
- 20 11. The method of claim 1, wherein said level of *CYP24* is detected by measuring the level of 25-hydroxy vitamin D3 24-hydroxylase enzyme activity in said biological sample, wherein an increased level of 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in said sample as compared to 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in said control sample indicates a predisposition to cancer.
- 25 12. The method of claim 11, wherein said level of 25-hydroxyvitamin D3 24-hydroxylase activity is measured in said biological sample and said control sample at the same vitamin D receptor activity or the activity levels are normalized to the level of vitamin D receptor activity in the sample and control.
13. The method of claim 1, wherein said animal is a mammal selected from the group consisting of humans, non-human primates, canines, felines, murines, bovines, equines, porcines, and lagomorphs.
- 30 14. The method of claim 1, wherein said biological sample is selected from the group consisting of excised tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, and urine.
- 35 15. The method of claim 1, wherein the difference between said increased level of *CYP24* in said biological sample and the level of *CYP24* in said control sample is a statistically significant difference.
16. The method of claim 1, wherein said increased level of *CYP24* in said biological sample is at least about 2-fold greater than the level of *CYP24* in said control sample.
- 40 17. The method of claim 1, wherein said increased level of *CYP24* in said biological sample is at least about 4-fold greater than said level of *CYP24* in said control sample.
- 45 18. A method of estimating the survival expectancy of an animal with cancer, said method comprising:
 - (i) using a biological sample from said animal;
 - (ii) detecting the level of *CYP24* within said biological sample; and
 - (iii) comparing said level of *CYP24* with the level of *CYP24* in a control sample taken from a normal, cancer-free tissue;
- 50 wherein an increased level of *CYP24* in said biological sample compared to the level of *CYP24* in said control sample indicates a reduced survival expectancy in said animal compared to in an animal with cancer that has a normal level of *CYP24*.
- 55 19. The method of claim 18, wherein said level of *CYP24* is detected by determining the copy number of *CYP24* genes in the cells of said animal.
20. The method of claim 19, wherein said copy number is determined by hybridization to an array of nucleic acid probes.

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21. The method of claim 19, wherein said copy number is measured using Comparative Genomic Hybridization.
22. The method of claim 21, wherein said Comparative Genomic hybridization is performed on an array.
- 5 23. The method of claim 18, wherein said level of *CYP24* is detected by measuring the level of *CYP24* mRNA in said biological sample, wherein an increased level of *CYP24* RNA in said sample as compared to *CYP24* RNA in said control sample indicates a reduced survival expectancy.
- 10 24. The method of claim 23, wherein said level of *CYP24* mRNA is measured in said biological sample and said control sample at the same vitamin D receptor activity or the activity levels are normalized to the level of vitamin D receptor activity in the sample and control.
- 15 25. The method of claim 18, wherein said level of *CYP24* is detected by measuring the level of *CYP24* protein in said biological sample, wherein an increased level of *CYP24* protein in said sample as compared to *CYP24* protein in said control sample, at a given level of vitamin D receptor activity indicates a reduced survival expectancy.
- 20 26. The method of claim 18, wherein said level of *CYP24* is detected by measuring the level of 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in said biological sample, wherein an increased level of 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in said sample as compared to 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in said control sample indicates a reduced survival expectancy.
- 25 27. The method of claim 26, wherein said level of 25-hydroxyvitamin D3 24-hydroxylase activity is measured in said biological sample and said control sample at the same vitamin D receptor activity or the activity levels are normalized to the level of vitamin D receptor activity in the sample and control.
28. The method of claim 18, wherein said animal is a mammal selected from the group consisting of humans, non-human primates, canines, felines, murines, bovines, equines, porcines, and lagomorphs.
- 30 29. The method of claim 18, wherein said biological sample is selected from the group consisting of excised tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, and urine.
- 30 30. The method of claim 18, wherein the difference between said increased level of *CYP24* in said biological sample and the level of *CYP24* in said control sample is a statistically significant difference.
- 35 31. The method of claim 18, wherein said increased level of *CYP24* in said biological sample is at least about 2-fold greater than the level of *CYP24* in said control sample.
32. The method of claim 18, wherein said increased level of *CYP24* in said biological sample is at least about 4-fold greater than the level of *CYP24* in said control sample.
- 40 33. A diagnostic method of selecting a cancer therapy in an animal, said method comprising:
- (i) using a biological sample from said animal;
 - (ii) detecting the level of *CYP24* within said biological sample;
 - 45 (iii) comparing said level of *CYP24* with a level of *CYP24* in a control sample from a normal, cancer-free tissue; and
 - (iv) selecting a cancer therapy in those animals having an increased level of *CYP24* compared to the level of *CYP24* in said control sample.
34. The method of claim 33, wherein said cancer therapy is selected from the group consisting of chemotherapy, radiation therapy, surgery, antihormone therapy, and immunotherapy.
- 50 35. The method of claim 34, wherein said cancer therapy is an adjuvant cancer therapy.
36. The method of claim 33, wherein said level of *CYP24* is detected by determining the copy number of *CYP24* genes in the cells of said animal.
- 55 37. The method of claim 36, wherein said copy number of *CYP24* genes is determined by hybridization to an array of nucleic acid probes.

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38. The method of claim 36, wherein said copy number of *CYP24* genes is measured using Comparative Genomic Hybridization (CGH).
39. The method of claim 38, wherein said Comparative Genomic Hybridization is performed on an array.
- 5 40. The method of claim 33, wherein said level of *CYP24* is detected by measuring the levels of *CYP24* mRNA in said biological sample, wherein an increased level of *CYP24* RNA in said sample as compared to *CYP24* RNA in said control sample indicates the need for an adjuvant cancer therapy.
- 10 41. The method of claim 40, wherein said level of *CYP24* RNA is measured in said biological sample and said control sample at the same vitamin D receptor activity or the activity levels are normalized to the level of vitamin D receptor activity in the sample and control.
- 15 42. The method of claim 33, wherein said level of *CYP24* is detected by measuring the level of *CYP24* protein in said biological sample, wherein an increased level of *CYP24* protein in said sample as compared to *CYP24* protein in said control sample indicates the need for an adjuvant cancer therapy.
- 20 43. The method of claim 42, wherein said level of *CYP24* protein activity is measured in said biological sample and said control sample at the same vitamin D receptor activity or the activity levels are to the level of vitamin D receptor activity in the sample and control.
- 25 44. The method of claim 33, wherein said *CYP24* level is detected by measuring the level of 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in said biological sample wherein an increased level of 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in said sample as compared to 25-hydroxyvitamin D3 24-hydroxylase enzyme activity in said control sample, at a given level of vitamin D receptor activity indicates the need for an adjuvant cancer therapy.
- 30 45. The method of claim 44, wherein said level 25-hydroxyvitamin D3 24-hydroxylase enzyme activity is measured in said biological sample and said control sample at the same vitamin D receptor activity or the activity levels are normalized to the level of vitamin D receptor activity in the sample and control.
- 35 46. The method of claim 33, wherein said animal is a mammal selected from the group consisting of humans, non-human primates, canines, felines, murines, bovines, equines, porcines, and lagomorphs.
- 40 47. The method of claim 33, wherein said biological sample is selected from the group consisting of excised tissue, whole blood, serum, plasma, cerebrospinal fluid, buccal scrape, saliva, and urine.
- 45 48. The method of claim 33, wherein the difference between said increased level of *CYP24* in said biological sample and the level of *CYP24* in said control sample is a statistically significant difference.
49. The method of claim 33, wherein said increased level of *CYP24* in said biological sample is at least about 2-fold greater than the level of *CYP24* in said control sample.
50. The method of claim 33, wherein said increased level of *CYP24* in said biological sample is at least about 4-fold greater than the level of *CYP24* in said control sample.

Patentansprüche

- 50 1. Verfahren zum Ermitteln einer Prädisposition für Krebs in einem tierischen Lebewesen, aufweisend die Schritte:
- (i) Verwenden einer biologischen Probe des tierischen Lebewesens;
(ii) Erfassen des *CYP24*-Spiegels in der biologischen Probe; und
(iii) Vergleichen des *CYP24*-Spiegels mit einem *CYP24*-Spiegel in einer Kontrollprobe, die einem normalen, kreisfreien Gewebe entnommen worden ist;
- 55 wobei ein verglichen mit dem *CYP24*-Spiegel in der Kontrollprobe erhöhter *CYP24*-Spiegel in der biologischen Probe eine Prädisposition für Krebs in dem tierischen Lebewesen anzeigt.

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2. Verfahren nach Anspruch 1, wobei der CYP24-Spiegel durch Bestimmung der Anzahl von CYP24-Genkopien in den Zellen der biologischen Probe erfasst wird.
- 5 3. Verfahren nach Anspruch 2, wobei die Anzahl von Genkopien mittels der Comparative Genomic Hybridization (CGH) gemessen wird.
4. Verfahren nach Anspruch 2, wobei die Anzahl von Genkopien durch Hybridisierung mit einem Array von Nukleinsäure-Sonden bestimmt wird.
- 10 5. Verfahren nach Anspruch 3, wobei die Comparative Genomic Hybridization auf einem Array durchgeführt wird.
6. Verfahren nach Anspruch 1, wobei der CYP24-Spiegel durch Messung des CYP24-mRNA-Spiegels in der biologischen Probe ermittelt wird,
wobei ein verglichen mit CYP24-RNA in der Kontrollprobe erhöhter CYP24-RNA-Spiegel in der Probe eine Prädisposition für Krebs anzeigt.
- 15 7. Verfahren nach Anspruch 6, wobei der CYP24-mRNA-Spiegel in der biologischen Probe und in der Kontrollprobe bei derselben Vitamin D-Rezeptoraktivität gemessen wird oder die CYP24-mRNA-Spiegel auf den Grad der Vitamin D-Rezeptoraktivität in der Probe und in der Kontrollprobe normalisiert werden.
- 20 8. Verfahren nach Anspruch 6, wobei der CYP24-mRNA-Spiegel durch Hybridisierung mit einer oder mit mehreren Sonden auf einem Array gemessen wird.
9. Verfahren nach Anspruch 1, wobei der CYP24-Spiegel durch Messung des CYP24-Protein-Spiegels in der biologischen Probe ermittelt wird,
wobei ein verglichen mit CYP24-Protein in der Kontrollprobe erhöhter CYP24-Protein-Spiegel in der Probe eine Prädisposition für Krebs anzeigt.
- 25 10. Verfahren nach Anspruch 9, wobei der CYP24-Protein-Spiegel in der biologischen Probe und der Kontrollprobe bei derselben Vitamin D-Rezeptoraktivität gemessen wird oder die Protein-Spiegel auf den Grad der Vitamin D-Rezeptoraktivität in der Probe und in der Kontrollprobe normalisiert werden.
- 30 11. Verfahren nach Anspruch 1, wobei der CYP24-Spiegel durch Messung des Grades der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der biologischen Probe ermittelt wird, wobei eine verglichen mit der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der Kontrollprobe erhöhte 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der Probe eine Prädisposition für Krebs anzeigt.
- 35 12. Verfahren nach Anspruch 11, wobei der Grad der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der biologischen Probe und in der Kontrollprobe bei derselben Vitamin D-Rezeptoraktivität gemessen wird oder die Aktivitätsgrade auf den Grad der Vitamin D-Rezeptoraktivität in der Probe und der Kontrollprobe normalisiert werden.
- 40 13. Verfahren nach Anspruch 1, wobei das tierische Lebewesen ein Säugetier ist, das aus der Gruppe bestehend aus Menschen, nichtmenschlichen Primaten, Hunden, Katzen, Mäusen, Rindern, Pferden, Schweinen und hasenartigen Tieren ausgewählt wird.
- 45 14. Verfahren nach Anspruch 1, wobei die biologische Probe aus einer Gruppe bestehend aus exzidiertem Gewebe, Vollblut, Serum, Plasma, bukkaler Abschabung, Speichel, Zerebrospinalflüssigkeit und Urin ausgewählt wird.
- 50 15. Verfahren nach Anspruch 1, wobei der Unterschied zwischen dem erhöhten CYP24-Spiegel in der biologischen Probe und dem CYP24-Spiegel in der Kontrollprobe ein statistisch signifikanter Unterschied ist.
16. Verfahren nach Anspruch 1, wobei der erhöhte CYP24-Spiegel in der biologischen Probe wenigstens zweimal so groß wie der CYP24- Spiegel in der Kontrollprobe ist.
- 55 17. Verfahren nach Anspruch 1, wobei der erhöhte CYP24- Spiegel in der biologischen Probe wenigstens viermal so groß wie der CYP24- Spiegel in der Kontrollprobe ist.
18. Verfahren zum Abschätzen der Überlebenserwartung eines tierischen Lebewesens mit Krebs, aufweisend die Schrit-

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te:

- (i) Verwenden einer biologischen Probe des tierischen Lebewesens;
- (ii) Erfassen des CYP24- Spiegels in der biologischen Probe; und
- (iii) Vergleichen des CYP24- Spiegels mit dem CYP24-Spiegel in einer Kontrollprobe, die einem normalen, krebsfreien Gewebe entnommen worden ist,

wobei ein verglichen mit dem CYP24-Spiegel in der Kontrollprobe erhöhter CYP24-Spiegel in der biologischen Probe eine verglichen mit einem tierischen Lebewesen mit Krebs, das einen normalen CYP24-Spiegel hat, verringerte Überlebenserwartung des tierischen Lebewesens anzeigt.

19. Verfahren nach Anspruch 18, wobei der CYP24-Spiegel durch Bestimmung der Anzahl von CYP24-Genkopien in den Zellen des tierischen Lebewesens ermittelt wird.
20. Verfahren nach Anspruch 19, wobei die Anzahl von Genkopien durch Hybridisierung mit einem Array von Nukleinsäure-Sonden bestimmt wird.
21. Verfahren nach Anspruch 19, wobei die Anzahl von Genkopien mittels der Comparative Genomic Hybridization gemessen wird.
22. Verfahren nach Anspruch 21, wobei die Comparative Genomic Hybridization auf einem Array durchgeführt wird.
23. Verfahren nach Anspruch 18, wobei der CYP24-Spiegel durch Messung des CYP24-mRNA-Spiegels in der biologischen Probe ermittelt wird, wobei ein verglichen mit CYP24-RNA in der Kontrollprobe erhöhter CYP24-RNA-Spiegel in der Probe eine verringerte Überlebenserwartung anzeigt.
24. Verfahren nach Anspruch 23, wobei der CYP24-mRNA-Spiegel in der biologischen Probe und in der Kontrollprobe bei derselben Vitamin D-Rezeptoraktivität gemessen wird oder die Aktivitätsgrade auf den Grad der Vitamin D-Rezeptoraktivität in der Probe und der Kontrollprobe normalisiert werden.
25. Verfahren nach Anspruch 18, wobei der CYP24-Spiegel durch Messung des CYP24-Protein-Spiegels in der biologischen Probe ermittelt wird, wobei ein verglichen bei einem gegebenen Grad der Vitamin D-Rezeptoraktivität mit CYP24-Protein in der Kontrollprobe erhöhter CYP24-Protein-Spiegel in der Probe eine verringerte Überlebenserwartung anzeigt.
26. Verfahren nach Anspruch 18, wobei der CYP24-Spiegel durch Messung des Grades der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der biologischen Probe ermittelt wird, wobei ein verglichen mit dem Grad der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der Kontrollprobe erhöhter Grad der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der Probe eine verringerte Überlebenserwartung anzeigt.
27. Verfahren nach Anspruch 26, wobei der Grad der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der biologischen Probe und in der Kontrollprobe bei derselben Vitamin D-Rezeptoraktivität gemessen wird oder die Aktivitätsgrade auf den Grad der Vitamin D-Rezeptoraktivität in der Probe und in der Kontrollprobe normalisiert werden.
28. Verfahren nach Anspruch 18, wobei das tierische Lebewesen ein Säugetier ist, das aus der Gruppe bestehend aus Menschen, nichtmenschlichen Primaten, Hunden, Katzen, Mäusen, Rindern, Pferden, Schweinen und hasenartigen Tieren ausgewählt wird.
29. Verfahren nach Anspruch 18, wobei die biologische Probe aus einer Gruppe bestehend aus exzidiertem Gewebe, Vollblut, Serum, Plasma, bukkaler Abschabung, Speichel, Zerebrospinalflüssigkeit und Urin ausgewählt wird.
30. Verfahren nach Anspruch 18, wobei der Unterschied zwischen dem erhöhten CYP24-Spiegel in der biologischen Probe und dem CYP24-Spiegel in der Kontrollprobe ein statistisch signifikanter Unterschied ist.
31. Verfahren nach Anspruch 18, wobei der erhöhte CYP24-Spiegel in der biologischen Probe wenigstens zweimal so groß wie der CYP24-Spiegel in der Kontrollprobe ist.

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32. Verfahren nach Anspruch 18, wobei der erhöhte CYP24-Spiegel in der biologischen Probe wenigstens viermal so groß wie der CYP24-Spiegel in der Kontrollprobe ist.
- 5 33. Diagnostisches Verfahren zum Auswählen einer Therapie für ein tierisches Lebewesen, aufweisend die Schritte:
- (i) Verwenden einer biologischen Probe des tierischen Lebewesens;
 - (ii) Erfassen der CYP24-Spiegels in der biologischen Probe;
 - (iii) Vergleichen des CYP24-Spiegels mit einem CYP24-Spiegel in einer Kontrollprobe eines normalen, krebsfreien Gewebes; und
 - 10 (iv) Auswählen einer Krebstherapie für die tierischen Lebewesen, die verglichen mit dem CYP24-Spiegel in der Kontrollprobe einen erhöhten CYP24-Spiegel haben.
34. Verfahren nach Anspruch 33, wobei die Krebstherapie aus der Gruppe bestehend aus Chemotherapie, Strahlentherapie, Chirurgie, Antihormontherapie und Immuntherapie ausgewählt wird.
- 15 35. Verfahren nach Anspruch 34, wobei die Krebstherapie eine unterstützende Krebstherapie ist.
36. Verfahren nach Anspruch 33, wobei der CYP24-Spiegel durch Bestimmung der Anzahl von CYP24-Genkopien in den Zellen des tierischen Lebewesens ermittelt wird.
- 20 37. Verfahren nach Anspruch 36, wobei die Anzahl von CYP24-Genkopien durch Hybridisierung mit einem Array von Nukleinsäure-Sonden bestimmt wird.
38. Verfahren nach Anspruch 36, wobei die Anzahl von CYP24-Genkopien mittels der Comparative Genomic Hybridization (CGH) gemessen wird.
- 25 39. Verfahren nach Anspruch 38, wobei die Comparative Genomic Hybridization auf einem Array durchgeführt wird.
40. Verfahren nach Anspruch 33, wobei der CYP24-Spiegel durch Messung des CYP24-mRNA-Spiegels in der biologischen Probe ermittelt wird, wobei ein verglichen mit CYP24-RNA in der Kontrollprobe erhöhter CYP24-RNA-Spiegel in der Probe das Erfordernis einer unterstützenden Krebstherapie anzeigt.
- 30 41. Verfahren nach Anspruch 40, wobei der CYP24-RNA-Spiegel in der biologischen Probe und in der Kontrollprobe bei derselben Vitamin D-Rezeptoraktivität gemessen wird oder die Aktivitätsgrade auf den Grad der Vitamin D-Rezeptoraktivität in der Probe und in der Kontrollprobe normalisiert werden.
- 35 42. Verfahren nach Anspruch 33, wobei der CYP24-Spiegel durch Messung des CYP24-Protein-Spiegels in der biologischen Probe ermittelt wird, wobei ein verglichen mit CYP24-Protein in der Kontrollprobe erhöhter CYP24-Protein-Spiegel in der Probe das Erfordernis einer unterstützenden Krebstherapie anzeigt.
- 40 43. Verfahren nach Anspruch 42, wobei der Grad der CYP24-Proteinaktivität in der biologischen Probe und in der Kontrollprobe bei derselben Vitamin D-Rezeptoraktivität gemessen wird oder die Aktivitätsgrade auf den Grad Vitamin D-Rezeptoraktivität in der Probe und der Kontrollprobe normalisiert werden.
- 45 44. Verfahren nach Anspruch 33, wobei der CYP24-Spiegel durch Messung des Grades der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der biologischen Probe ermittelt wird, wobei ein verglichen bei einem gegebenen Grad der Vitamin D-Rezeptoraktivität mit dem Grad der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der Kontrollprobe erhöhter Grad der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der Probe das Erfordernis einer unterstützenden Krebstherapie anzeigt.
- 50 45. Verfahren nach Anspruch 44, wobei der Grad der 25-Hydroxyvitamin D3-24-Hydroxylase-Enzymaktivität in der biologischen Probe und in der Kontrollprobe bei derselben Vitamin D-Rezeptoraktivität gemessen wird oder die Aktivitätsgrade auf den Grad der Vitamin D-Rezeptoraktivität in der Probe und in der Kontrollprobe normalisiert werden.
- 55 46. Verfahren nach Anspruch 33, wobei das tierische Lebewesen ein Säugetier ist, das aus der Gruppe bestehend aus Menschen, nichtmenschlichen Primaten, Hunden, Katzen, Mäusen, Rindern, Pferden, Schweinen und hasenartigen Tieren ausgewählt wird.

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47. Verfahren nach Anspruch 33, wobei die biologische Probe aus einer Gruppe bestehend aus exzidiertem Gewebe, Vollblut, Serum, Plasma, bukkaler Abschabung, Speichel, Zerebrospinalflüssigkeit und Urin ausgewählt wird.
- 5 48. Verfahren nach Anspruch 33, wobei der Unterschied zwischen dem erhöhten CYP24-Spiegel in der biologischen Probe und dem CYP24-Spiegel in der Kontrollprobe ein statistisch signifikanter Unterschied ist.
49. Verfahren nach Anspruch 33, wobei der erhöhte CYP24-Spiegel in der biologischen Probe wenigstens zweimal so groß wie der CYP24-Spiegel in der Kontrollprobe ist.
- 10 50. Verfahren nach Anspruch 33, wobei der erhöhte CYP24-Spiegel in der biologischen Probe wenigstens viermal so groß wie der CYP24-Spiegel in der Kontrollprobe ist.

Revendications

- 15 1. Procédé pour la détection d'une prédisposition au cancer chez un animal, ledit procédé comprenant:

- (i) l'utilisation d'un échantillon biologique provenant dudit animal;
20 (ii) la détection du taux de CYP24 dans ledit échantillon biologique; et
20 (iii) la comparaison dudit taux de CYP24 avec un taux de CYP24 dans un échantillon témoin prélevé à partir d'un tissu normal, exempt de cancer;

tandis qu'un taux accru de CYP24 dans ledit échantillon biologique par comparaison avec le taux de CYP24 dans ledit échantillon témoin indique une prédisposition au cancer chez ledit animal.

- 25 2. Procédé selon la revendication 1, dans lequel ledit taux de CYP24 est détecté par détermination du nombre de copies des gènes CYP24 dans les cellules dudit échantillon biologique.
- 30 3. Procédé selon la revendication 2, dans lequel ledit nombre de copies est mesuré au moyen d'une Hybridation Génomique Comparative (CGH).
4. Procédé selon la revendication 2, dans lequel ledit nombre de copies est déterminé par hybridation à un arrangement de sondes d'acide nucléique.
- 35 5. Procédé selon la revendication 3, dans lequel ladite Hybridation Génomique Comparative est réalisée sur un arrangement.
6. Procédé selon la revendication 1, dans lequel ledit taux de CYP24 est détecté par mesure du taux d'ARNm de CYP24 dans ledit échantillon biologique, tandis qu'un taux accru d'ARN de CYP24 dans ledit échantillon par comparaison avec l'ARN de CYP24 dans ledit échantillon témoin indique une prédisposition au cancer.
- 40 7. Procédé selon la revendication 6, dans lequel ledit taux d'ARNm de CYP24 est mesuré dans ledit échantillon biologique et ledit échantillon témoin pour la même activité du récepteur de la vitamine D, ou bien les taux d'ARNm de CYP24 sont normalisés au taux d'activité du récepteur de la vitamine D dans l'échantillon et le témoin.
- 45 8. Procédé selon la revendication 6, dans lequel ledit taux d'ARNm de CYP24 est mesuré par hybridation à une ou plusieurs sondes sur un arrangement.
9. Procédé selon la revendication 1, dans lequel ledit taux de CYP24 est détecté par mesure du taux de protéine CYP24 dans ledit échantillon biologique, tandis qu'un taux accru de protéine CYP24 dans ledit échantillon par comparaison avec la protéine CYP24 dans ledit échantillon témoin indique une prédisposition au cancer.
- 50 10. Procédé selon la revendication 9, dans lequel le taux de protéine CYP24 est mesuré dans l'échantillon biologique et l'échantillon témoin pour la même activité de récepteur de la vitamine D, ou bien les taux de protéine sont normalisés au taux d'activité du récepteur de la vitamine D dans l'échantillon et le témoin.
- 55 11. Procédé selon la revendication 1, dans lequel ledit taux de CYP24 est détecté par mesure du niveau d'activité de l'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon biologique, tandis qu'un niveau accru d'ac-

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tivité d'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon par comparaison avec l'activité d'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon témoin indique une prédisposition au cancer.

- 5
12. Procédé selon la revendication 11, dans lequel ladite activité de 25-hydroxyvitamine D3 24-hydroxylase est mesurée dans ledit échantillon biologique et ledit échantillon témoin pour la même activité de récepteur de vitamine D, ou bien les niveaux d'activité sont normalisés par rapport au niveau d'activité de récepteur de vitamine D dans l'échantillon et le témoin.
- 10
13. Procédé selon la revendication 1, dans lequel ledit animal est un mammifère choisi dans le groupe consistant en les espèces humaines, primates non-humaines, canines, félines, murines, bovines, équines, porcines et lagomorphes.
- 15
14. Procédé selon la revendication 1, dans lequel ledit échantillon biologique est choisi dans le groupe consistant en tissu excisé, sang total, sérum, plasma, frottis buccal, salive, liquide céphalo-rachidien, et urine.
- 20
15. Procédé selon la revendication 1, dans lequel la différence entre ledit taux accru de *CYP24* dans ledit échantillon biologique et le taux de *CYP24* dans ledit échantillon témoin est une différence statistiquement significative.
- 25
16. Procédé selon la revendication 1, dans lequel ledit taux accru de *CYP24* dans ledit échantillon biologique est au moins environ 2 fois plus élevé que le taux de *CYP24* dans ledit échantillon témoin.
- 30
17. Procédé selon la revendication 1, dans lequel ledit taux accru de *CYP24* dans ledit échantillon biologique est au moins environ 4 fois plus élevé que ledit taux de *CYP24* dans ledit échantillon témoin.
- 35
18. Procédé pour l'estimation de l'espérance de survie d'un animal ayant un cancer, ledit procédé comprenant:
- (i) l'utilisation d'un échantillon biologique provenant dudit animal;
 - (ii) la détection du taux de *CYP24* dans ledit échantillon biologique; et
 - (iii) la comparaison dudit taux de *CYP24* avec le taux de *CYP24* dans un échantillon témoin prélevé à partir d'un tissu normal, exempt de cancer;
- tandis qu'un taux accru de *CYP24* dans ledit échantillon biologique par comparaison avec le taux de *CYP24* dans ledit échantillon témoin indique une espérance de survie réduite chez ledit animal par comparaison avec le cas d'un animal ayant un cancer qui a un taux normal de *CYP24*.
- 40
19. Procédé selon la revendication 18, dans lequel ledit taux de *CYP24* est détecté par détermination du nombre de copies de gènes *CYP24* dans les cellules dudit animal.
- 45
20. Procédé selon la revendication 19, dans lequel ledit nombre de copies est déterminé par hybridation à un arrangement de sondes d'acide nucléique.
- 50
21. Procédé selon la revendication 19, dans lequel ledit nombre de copies est mesuré au moyen d'une Hybridation Génomique Comparative.
- 55
22. Procédé selon la revendication 21, dans lequel ladite Hybridation Génomique Comparative est réalisée sur un arrangement.
23. Procédé selon la revendication 18, dans lequel ledit taux de *CYP24* est détecté par mesure du taux d'ARNm de *CYP24* dans ledit échantillon biologique, tandis qu'un taux accru d'ARN de *CYP24* dans ledit échantillon par comparaison avec l'ARN de *CYP24* dans ledit échantillon témoin indique une espérance de survie réduite.
24. Procédé selon la revendication 23, dans lequel ledit taux d'ARNm de *CYP24* est mesuré dans ledit échantillon biologique et ledit échantillon témoin pour la même activité de récepteur de la vitamine D, ou bien les niveaux d'activité sont normalisés sur le niveau d'activité de récepteur de la vitamine D dans l'échantillon et le témoin.
25. Procédé selon la revendication 18, dans lequel ledit taux de *CYP24* est détecté par mesure du taux de protéine *CYP24* dans ledit échantillon biologique, tandis qu'un taux accru de protéine *CYP24* dans ledit échantillon par comparaison avec la protéine *CYP24* dans ledit échantillon témoin, à un niveau donné d'activité de récepteur de

la vitamine D, indique une espérance de survie réduite.

- 5 26. Procédé selon la revendication 18, dans lequel ledit taux de *CYP24* est détecté par mesure du niveau d'activité d'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon biologique, tandis qu'un niveau accru d'activité d'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon par comparaison avec l'activité d'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon témoin indique une espérance de survie réduite.
- 10 27. Procédé selon la revendication 26, dans lequel ledit niveau d'activité de 25-hydroxyvitamine D3 24-hydroxylase est mesuré dans ledit échantillon biologique et ledit échantillon témoin pour la même activité de récepteur de la vitamine D, ou bien les niveaux d'activité sont normalisés sur le niveau d'activité de récepteur de la vitamine D dans l'échantillon et le témoin.
- 15 28. Procédé selon la revendication 18, dans lequel ledit animal est un mammifère choisi dans le groupe consistant en les espèces humaines, primates non-humaines, canines, félines, murines, bovines, équines, porcines et lagomorphes.
- 20 29. Procédé selon la revendication 18, dans lequel ledit échantillon biologique est choisi dans le groupe consistant en tissu excisé, sang total, sérum, plasma, frottis buccal, salive, liquide céphalo-rachidien, et urine.
- 25 30. Procédé selon la revendication 18, dans lequel la différence entre ledit taux accru de *CYP24* dans ledit échantillon biologique et le taux de *CYP24* dans ledit échantillon témoin est une différence statistiquement significative.
- 30 31. Procédé selon la revendication 18, dans lequel ledit taux accru de *CYP24* dans ledit échantillon biologique est au moins environ 2 fois plus élevé que le taux de *CYP24* dans ledit échantillon témoin.
- 35 32. Procédé selon la revendication 18, dans lequel ledit taux accru de *CYP24* dans ledit échantillon biologique est au moins environ 4 fois plus élevé que le taux de *CYP24* dans ledit échantillon témoin.
- 30 33. Procédé de diagnostic pour la sélection d'une thérapie cancéreuse chez un animal, ledit procédé comprenant:
- (i) l'utilisation d'un échantillon biologique provenant dudit animal;
 - (ii) la détection du taux de *CYP24* dans ledit échantillon biologique;
 - (iii) la comparaison dudit taux de *CYP24* avec un taux de *CYP24* dans un échantillon témoin prélevé à partir d'un tissu normal, exempt de cancer; et
 - (iv) la sélection de la thérapie cancéreuse chez les animaux ayant un taux accru de *CYP24* par comparaison avec le taux de *CYP24* dans ledit échantillon témoin.
- 40 34. Procédé selon la revendication 33, dans lequel ladite thérapie cancéreuse est choisie dans le groupe consistant en chimiothérapie, thérapie par irradiation, chirurgie, thérapie anti-hormonale, et immunothérapie.
- 45 35. Procédé selon la revendication 34, dans lequel ladite thérapie cancéreuse est une thérapie cancéreuse d'appoint.
36. Procédé selon la revendication 33, dans lequel ledit taux de *CYP24* est détecté par détermination du nombre de copies des gènes *CYP24* dans les cellules dudit animal.
37. Procédé selon la revendication 36, dans lequel ledit nombre de copies de gènes *CYP24* est déterminé par hybridation sur un arrangement de sondes d'acide nucléique.
- 50 38. Procédé selon la revendication 36, dans lequel ledit nombre de copies de gènes *CYP24* est mesuré par Hybridation Génomique Comparative (CGH).
39. Procédé selon la revendication 38, dans lequel ladite Hybridation Génomique Comparative est réalisée sur un arrangement.
- 55 40. Procédé selon la revendication 33, dans lequel ledit taux de *CYP24* est détecté par mesure des taux d'ARNm de *CYP24* dans ledit échantillon biologique, tandis qu'un taux accru d'ARN de *CYP24* dans ledit échantillon par comparaison avec l'ARN de *CYP24* dans ledit échantillon témoin indique le besoin d'une thérapie cancéreuse d'appoint.

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41. Procédé selon la revendication 40, dans lequel ledit taux d'ARN de *CYP24* est mesuré dans ledit échantillon biologique et ledit échantillon témoin pour la même activité du récepteur de la vitamine D, ou bien les niveaux d'activité sont normalisés par rapport au niveau d'activité du récepteur de la vitamine D dans l'échantillon et le témoin.
- 5 42. Procédé selon la revendication 33, dans lequel ledit taux de *CYP24* est détecté par mesure du taux de protéine *CYP24* dans ledit échantillon biologique, tandis qu'un taux accru de protéine *CYP24* dans ledit échantillon par comparaison avec la protéine *CYP24* dans ledit échantillon témoin indique le besoin d'une thérapie cancéreuse d'appoint.
- 10 43. Procédé selon la revendication 42, dans lequel ledit taux d'activité de protéine *CYP24* est mesuré dans ledit échantillon biologique et ledit échantillon témoin pour la même activité de récepteur de la vitamine D, ou bien les niveaux d'activité sont normalisés par rapport au niveau d'activité du récepteur de la vitamine D dans l'échantillon et le témoin.
- 15 44. Procédé selon la revendication 33, dans lequel ledit taux de *CYP24* est détecté par mesure du taux d'activité de l'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon biologique, tandis qu'un taux accru d'activité d'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon par comparaison avec l'activité d'enzyme 25-hydroxyvitamine D3 24-hydroxylase dans ledit échantillon témoin, pour un niveau donné d'activité de récepteur de la vitamine D, indique le besoin d'une thérapie cancéreuse d'appoint.
- 20 45. Procédé selon la revendication 44, dans lequel ladite activité de 25-hydroxyvitamine D3 24-hydroxylase est mesurée dans ledit échantillon biologique et ledit échantillon témoin pour la même activité de récepteur de vitamine D, ou bien les niveaux d'activité sont normalisés par rapport au niveau d'activité de récepteur de vitamine D dans l'échantillon et le témoin.
- 25 46. Procédé selon la revendication 33, dans lequel ledit animal est un mammifère choisi dans le groupe consistant en les espèces humaines, primates non-humaines, canines, félines, murines, bovines, équines, porcines et lagomorphes.
- 30 47. Procédé selon la revendication 33, dans lequel ledit échantillon biologique est choisi dans le groupe consistant en tissu excisé, sang total, sérum, plasma, liquide céphalo-rachidien, frottis buccal, salive, et urine.
48. Procédé selon la revendication 33, dans lequel la différence entre ledit taux accru de *CYP24* dans ledit échantillon biologique et le taux de *CYP24* dans ledit échantillon témoin est une différence statistiquement significative.
- 35 49. Procédé selon la revendication 33, dans lequel ledit taux accru de *CYP24* dans ledit échantillon biologique est au moins environ 2 fois plus élevé que le taux de *CYP24* dans ledit échantillon témoin.
- 40 50. Procédé selon la revendication 33, dans lequel ledit taux accru de *CYP24* dans ledit échantillon biologique est au moins environ 4 fois plus élevé que le taux de *CYP24* dans ledit échantillon témoin.

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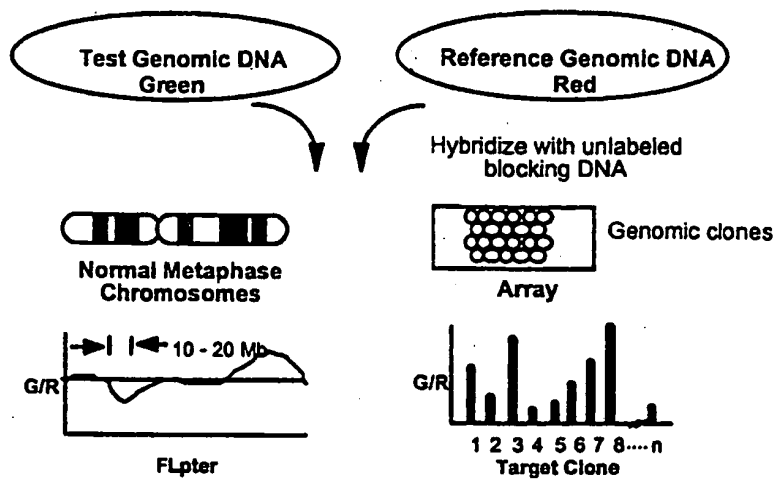


Fig. 1

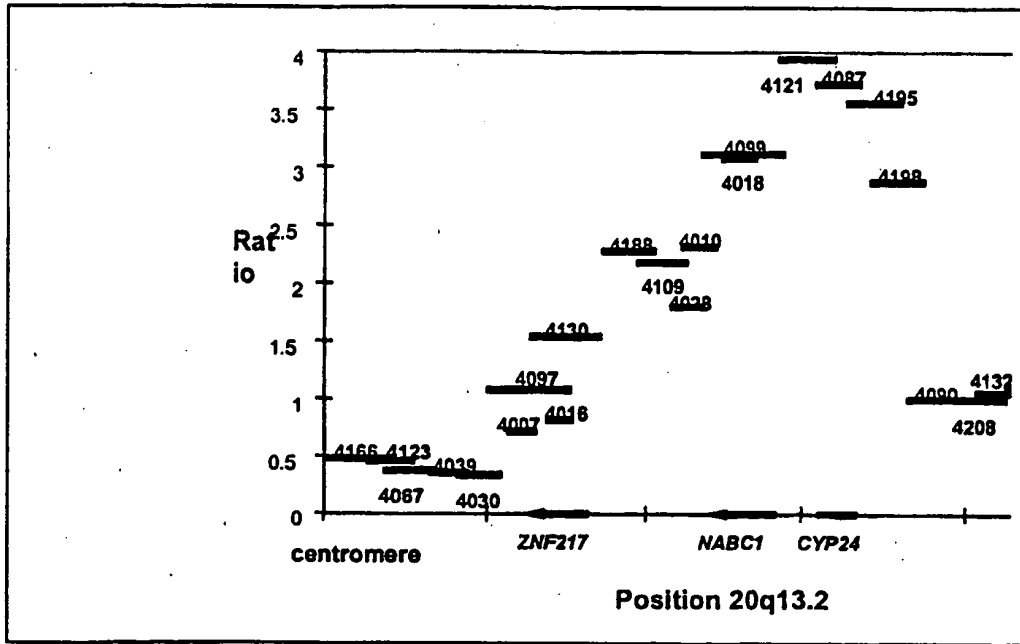


Fig. 2

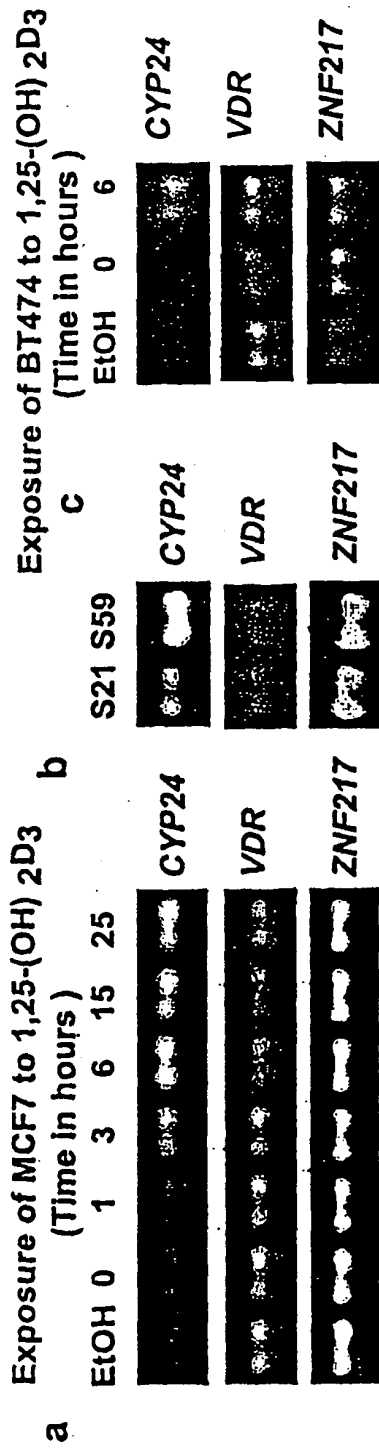


Figure 3

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	\$ i (CYP24) 的放大及其用途		
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摘要(译)

本发明涉及发现CYP24基因的扩增或CYP24活性的增加是癌症(例如,乳腺癌)的存在,进展或易感性的标志。使用该信息,本发明提供了检测动物癌症易感性的方法。该方法包括(i)提供来自动物(例如人类患者)的生物样品;(ii)检测生物样品中CYP24的水平;(iii)将CYP24水平与取自正常无癌组织的对照样品中的CYP24水平进行比较,其中生物样品中CYP24水平与对照样品中CYP24水平相比表明存在所述动物中的所述癌症。