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(54) Title: METHOD FOR DETERMINING PROGRESSION OF DUCTAL CARCINOMA IN SITU TO INVASIVE BREAST CANCER

(57) Abstract: The invention encompasses methods of diagnosing and monitoring subjects afflicted with ductal carcinoma in situ (DCIS) for the development of invasive ductal carcinoma (IDC) based on CAPER expression in breast tissue.



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METHOD FOR DETERMINING PROGRESSION OF DUCTAL CARCINOMA IN SITU TO INVASIVE BREAST CANCER

Field of the Invention

5 The invention relates to the diagnosis of invasive ductal carcinoma (IDC) through detecting elevated expression of CAPER in a breast lesion. The invention also relates to the monitoring of subjects for the development of IDC, through evaluation of CAPER expression in breast lesions.

Background of the Invention

10 Ductal carcinoma in situ (DCIS) is the most common kind of non-invasive breast cancer, with about 60,000 cases diagnosed in the United States each year (American Cancer Society). DCIS is a pre-malignant lesion in breast tissue that is non-invasive and does not spread beyond the milk duct to lymph nodes or other
15 organs. DCIS does, however, have a high rate of transformation to invasive breast cancer: about 25-50% of individuals diagnosed with DCIS eventually develop a more invasive cancer within 5 to 10 years after a DCIS diagnosis. Thus treatment of DCIS is aimed at reducing the risk of developing invasive breast cancer later on.

20 Treatment options for DCIS comprise from lumpectomy or mastectomy in combination with anti-estrogen therapy depending upon the extent and type of DCIS. To substantially reduce the risk of developing an invasive cancer, additional radiation therapy is often prescribed. However, not every woman benefits from mastectomy and radiation therapy. Given that not every woman diagnosed with DCIS goes on to develop IDC, the ability to clearly and rapidly identify those women with DCIS who
25 develop IDC and treat them accordingly is imperative. Of equal important is sparing women who will not benefit from aggressive treatment. However, knowledge of the molecular mechanisms that underlie the transformation of pre-malignant conditions such as DCIS to a more invasive breast cancer such as IDC is incomplete.

30 Coactivator of Activating Protein-1 and Estrogen Receptors (CAPER) is a recently identified, novel co-activator of nuclear receptors, including estrogen (ER- α and - β) and progesterone receptors (PR) (Jung et al., 2002, J. Biol. Chem. 277:1229-1234; Dowhan et al., 2005, Mol. Cell 17:429-439). CAPER was cloned based on its interaction with another nuclear co-activator gene, namely ASC-2 (Jung

et al., 2002, J. Biol. Chem. 277:1229-1234). CAPER is identical to Rbm39/HCC1, which was first identified as a factor that facilitates pre-mRNA processing/splicing (Jung et al., 2002, J. Biol. Chem. 277:1229-1234; Imai et al., 1993, J. Clin. Invest. 92:2419-2426; Ellis et al., 2008, J. Cell Biol. 181:921-934). Thus, CAPER may
5 functionally couple transcription with pre-mRNA splicing.

Identification of informative biomarkers for the early detection of invasive breast cancer are urgently needed as are simple and quick assays in order to identify an individual who's premalignant breast lesion has transformed to a more invasive cancer. The present invention meets this need.

Summary of the Invention

The invention is based on the discovery that CAPER expression is enhanced in breast tissue affected by invasive ductal carcinoma (IDC) relative to CAPER expression in breast tissue affected only by a pre-malignant condition, such
15 as ductal carcinoma in situ (DCIS).

The invention thus provides a method for determining the progression of a disease state from Ductal carcinoma in situ (DCIS) to Invasive Ductal Carcinoma (IDC) in a human subject, comprising determining the level of CAPER expression in a test sample and comparing the level of CAPER expression determined in the test
20 sample to the level of CAPER expression determined in a DCIS control sample. Elevated levels of CAPER expression in the test sample relative to the level of CAPER expression in said control sample indicates that the disease state of the subject has progressed from DCIS to IDC.

The invention also provides a method of monitoring the progression of a disease state from Ductal carcinoma in situ (DCIS) to Invasive Ductal Carcinoma (IDC) in a human subject, said method comprising determining the levels of CAPER expression in two samples obtained for at least two different time point. An increase in the level of CAPER expression in the sample obtained at the later time point either relative to an earlier sample or to a DCIS control sample indicates that the disease
30 state of the subject has progressed from DCIS to IDC.

Abbreviations

The following abbreviations are used herein:

DCIS: ductal carcinoma in situ

ELISA: enzyme linked immunosorbent assay

5 H & E: hematoxylin and eosin

IDC: invasive ductal carcinoma

RIA: radioimmunoassay

Brief Description of the Figures

10 For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1A depicts representative images of normal breast tissue (Normal) and
15 breast tissue afflicted with various stages of invasive ductal carcinoma (IDC) assayed for CAPER and Cytokeratin protein expression using immunohistochemistry. IDC1 = well differentiated IDC, IDC2 = moderately differentiated IDC; IDC3 = poorly differentiated IDC also designated 1, 2, and 3, respectively, on the X-axes of in Figures 1B and 1C.

20 Figures 1B depicts the distribution and mean AQUA[®] scores for CAPER protein expression in normal and IDC tissue using PM2000 AQUA automated quantitative analysis system. X-axis legends designate the source of the tissue samples: Normal (non-cancerous breast tissue), 1 (tissue afflicted with well differentiated IDC), 2 (tissue afflicted with moderately differentiated IDC), and 3
25 (tissue afflicted with poorly differentiated IDC).

Figure 1C depicts the mean AQUA scores for CAPER protein expression in normal and IDC tissue using PM2000 AQUA automated quatitative analysis system. X-axis legends designate the source of the tissue samples: Normal (non-cancerous breast tissue), 1 (tissue afflicted with well differentiated IDC), 2 (tissue afflicted with
30 moderately differentiated IDC), and 3 (tissue afflicted with poorly differentiated IDC). Error bars represent standard deviation. * = $p < 0.05$.

Figure 2 shows the mean relative CAPER mRNA copy number in pooled DCIS and IDC samples. Error bars represent standard deviation. IDC samples were

obtained from breast tissue that was stages as either IDC stage 1, IDC stage 2, IDC stage 3, or IDC stage 4.

Detailed Description of the Invention

5 The invention is based on the discovery that CAPER expression is enhanced in breast tissue affected by invasive ductal carcinoma (IDC) relative to CAPER expression in breast tissue affected only by a pre-malignant condition, such as ductal carcinoma in situ (DCIS). The nucleotide sequence of CAPER mRNA, and the encoded amino acid sequence of CAPER polypeptide are set forth in SEQ ID NO: 1
10 and SEQ ID NO; 2, respectively.

A. Breast Cancer

Breast cancer proceeds through discrete premalignant and malignant cellular stages: normal ductal epithelium, atypical ductal hyperplasia, ductal carcinoma in situ
15 (DCIS) including low grade DCIS and high grade DCIS, and finally invasive ductal carcinoma. The breast condition (especially breast pre-cancer or cancer) can be identified by mammogram, physical exam of the breast, MRI (magnetic resonance imaging), analysis of nipple aspirate fluid for abnormal cells, ductal lavage of the breast (e.g., as described in WO 1999/055384), biopsy, or other means known in the
20 art to identify the condition. Preferably the method of identifying the condition can provide information leading to which breast duct or ducts the condition is localized.

A "disease" is an abnormal condition of an organism, preferably a human, that impairs normal bodily function. Disease causes symptoms including pain and clinical dysfunction, specific to the affected organs or tissues of the body.

25 The term "cancer" is a class of diseases in which a group of cells display uncontrolled and progressive multiplication, invasion of adjacent issues, and sometimes metastasis.

The term "breast cancer" as used herein is defined as cancer which originates in the breast.

30 A "carcinoma" is a cancer that arises from epithelial cells.

A "lesion" as used herein refers to any abnormal tissue found in a breast. A breast lesion may be cancerous or non-cancerous.

The term "pre-malignant lesion" as used herein refers to a collection of cells in a breast with histopathological characteristics which suggest at least one of the cells has an increased risk of becoming breast cancer. In a preferred embodiment, the pre-malignant lesion is ductal carcinoma in situ (DCIS). In a specific embodiment, the collection of cells is a lump, tumor, mass, bump, bulge, swelling, and the like. Other terms in the art which are interchangeable with "pre-malignant lesion" include pre-malignant hyperplasia, pre-malignant neoplasia, and the like.

"Disease state of a lesion" refers to pathophysiological changes that occur in a lesion as a result of disease progression or treatment.

"Disease state of a subject" refers to the health status of a subject afflicted by a disease. The disease state of a subject may change based on disease progression or effective therapeutic intervention.

Invasive ductal carcinoma (IDC) is a malignant carcinoma that can invade surrounding tissue and organs and may metastasize to lymph nodes or other distal sites. Cancer stage is determined by a skilled artisan based on the size of the tumor, whether the cancer is invasive or non-invasive, whether lymph nodes are involved, and whether the cancer has spread beyond the breast. Stage 0 breast cancers are non-invasive, like DCIS. Stage one cancers are invasive, but small and localized (e.g. stage 1 IDC or IDC1). Stage two and three breast cancers typically are locally advanced and/or have spread to local lymph nodes, and possibly to the chest wall and/or skin of the breast (e.g. stage 2 or 3 IDC or IDC2 or IDC3). Stage four breast cancer is metastatic, most commonly spreading to either the lungs, liver, bones, or brain (e.g. stage 4 IDC or IDC4). Details of this staging system are further provided in: The International Union against Cancer details the TNM staging system (Tumour/Nodes/Metastasis), (UICC-TNM Classification of malignant tumours. Edited by L. H. Sobin and C. H. Wittekind. 5th Edition. New York: Wiley-Liss; 1997)

Ductal carcinoma in situ (DCIS), is a pre-malignant carcinoma in which some cytological signs of malignancy are present, but there is no histological evidence of invasion through the epithelial basement membrane. DCIS is typically identified during routine mammography as a microcalcification, a shadow, a lump, or other breast lesion present in the affected breast. DCIS comprises a heterogeneous group of histopathologic lesions that have been classified into several subtypes based primarily

on histological architectural pattern: micropapillary, papillary, solid, cribriform, and comedo.

DCIS is a stage 0 breast cancer that can be further graded as either low, moderate, or high grade by histological examination of a breast tissue sample by a skilled artisan using standard techniques known in the art. Low- and moderate-grade DCIS comprise relatively normal looking cells that are growing slowly. The affected duct may be completely filled with cancer cells (solid DCIS), have gaps between cancer cells (cribriform DCIS), or have cancer cells arranged in a fern-like pattern within the duct (papillary DCIS). High grade DCIS comprises comedo-type DCIS which presents histologically with high-grade nuclei, pleomorphism, and abundant central luminal necrosis.

B. CAPER expression in breast tissue

By “expression” is meant the process by which information from a gene is made into a functional gene product, such as RNA or protein. Thus, the “level of expression” of a gene product such as CAPER in a sample of interest, refers to the level of RNA, particularly the level of mRNA, or the level of the encoded protein, and is not intended to be limited to either.

An “elevated” CAPER expression in breast tissue obtained from a human subject means that the amount of CAPER protein, such as SEQ ID NO. 2, or the amount of nucleic acid encoding CAPER protein, e.g. SEQ ID NO. 1, measured in a test sample is greater than in a control sample. The level of CAPER mRNA or protein may be expressed either qualitatively or quantitatively. Quantitative description of enhanced CAPER expression may be expressed when per unit volume or unit mass of CAPER protein or nucleic acid is greater in the subject’s test sample than in the control sample. The level of CAPER protein or nucleic acid may be expressed in absolute units such as units of mass per unit volume (e.g. as nanograms per microlitre) or as numbers of nucleic acid, e.g. mRNA copy number. The level of CAPER expression may also be expressed in arbitrary units as determined relative to a control sample.

While any elevated level of CAPER expression may be indicative of disease, according to one embodiment, the level of CAPER expression is reflected in the mRNA copy number in the sample. In the practice of this invention, the level of

CAPER mRNA copy number in a subject's breast tissue sample is at least 2-fold greater than the CAPER mRNA copy number in the control.

In another embodiment, the level of CAPER expression is reflected by the amount of CAPER protein present in the sample.

5 As used herein, a "subject" is any human suspected of having IDC or at risk for developing IDC. As used herein, the term "subject at risk for IDC" refers to a subject with one or more risk factors for developing IDC. Risk factors include, but are not limited to, gender, age, genetic predisposition, environmental exposure, previous incidents of cancer, DCIS, preexisting non-cancer diseases, and lifestyle.

10 As used herein, the term "subject diagnosed with" or "afflicted with" a certain disease or condition refers to a subject who has been tested and found to have that disease or condition. In a preferred embodiment, the subject may be diagnosed with or afflicted with DCIS or IDC. The cancer may be diagnosed using any suitable method, including but not limited to, biopsy, x-ray, ultrasound, and the diagnostic
15 methods of the present invention.

As used herein, a "breast tissue sample" is any sample of cells or tissue obtained from a breast. A breast tissue sample comprising cells or tissue may be obtained by any method known in the art, including, but by no means limited to, a fine-needle aspiration biopsy, a core needle biopsy, a stereotactic biopsy, or an open
20 biopsy. It will be understood that a breast tissue sample may comprise multiple cell types and other components, such as blood components. As used herein, "breast tissue samples" includes controls or control samples.

As used herein, a "control," "control sample," or "DCIS control sample" refers to one or more breast tissue samples obtained from a DCIS lesion which is/are
25 negative for IDC at the time the sample was obtained.

In one embodiment, the control sample may comprise a pooled sample containing breast tissue samples obtained from a population of individuals where those samples have been identified as positive for DCIS, but negative for IDC. It is understood that when the control sample is obtained from multiple breast tissue
30 samples, the CAPER expression level can be expressed as an arithmetic mean, median, mode, or other suitable statistical measure of CAPER expression level measured in each sample. Multiple control samples may be pooled, and the CAPER

expression level of the pooled samples may be compared to the subject's breast tissue sample.

In another embodiment, a control sample may be obtained from a single individual afflicted with DCIS. The control sample from an individual may be obtained at the same or at a different time that a test sample is obtained. The control sample may be taken from the same lesion as the test sample, or a different lesion from the test sample.

As used herein, a "test sample" refers to one or more breast tissue samples taken from an individual for comparison to a control sample. A test sample may be a sample obtained from the same individual as the control. In another embodiment, a test sample may be obtained from a different individual as the control sample.

B.1. Detection of CAPER protein

In some embodiments, gene expression of CAPER is detected by determining the level of the corresponding protein or polypeptide in a breast tissue sample. Protein expression may be determined by any suitable method. Methods for determining the level of CAPER protein expression in a sample are well known in the art and include but are not limited to western blots, ELISA, immunoprecipitation, immunofluorescence, flow cytometry, and immunocytochemistry. In particular embodiments, CAPER protein level may be detected using, for example, antibodies that are directed against CAPER protein, or a fragment thereof. These antibodies can be used in various methods such as Western blot, ELISA, immunoprecipitation, or immunocytochemistry techniques. The invention is not limited to any one method of protein detection or quantification recited herein, but rather encompasses all presently known or heretofor unknown methods, such as methods that are discovered in the art.

In one embodiment CAPER protein levels are determined in a breast tissue sample. Assays based on CAPER protein-specific biomolecule interaction, include but are not limited to antibody-based assays, aptamer-based assays, receptor and ligand assays, enzyme activity assays, and allosteric regulator binding assays. Techniques for detecting protein concentrations in a sample are within the skill in the art. Preferably, CAPER protein levels are detected by immunoassays such as Western blot analysis, radioimmunoassay (RIA), immunofluorescent assay, chemiluminescent assay, or enzyme-linked immunosorbent assay (ELISA). Immunoassays suitable for

use in the present methods are described, for example, U.S. Pat. Nos. 5,976,809; 5,965,379; 5,571,680; 5,279,956; and 6,579,684, the entire disclosures of which are herein incorporated by reference. Detection of CAPER protein expression in histological samples using primary antibodies directed against CAPER protein or fragments thereof are presented throughout the Examples below, wherein said antibodies specifically bind a CAPER protein, polypeptide, or fragment thereof.

“Specifically binds” as used herein refers to antibody binding to a predetermined antigen with a preference that enables the antibody to be used to distinguish the antigen from others to an extent that permits the diagnostic assays described herein. Specific binding to CAPER means that the antibody preferentially binds CAPER versus other proteins.

The antibody used to detect CAPER protein expression in a breast tissue sample in an immunoassay can comprise a polyclonal or monoclonal antibody. The antibody can comprise an intact antibody, or antibody fragments capable of specifically binding a CAPER protein. Such fragments include, but are not limited to, Fab and F(ab')₂ fragments. Thus, as used herein, the term “antibody” includes both polyclonal and monoclonal antibodies. The term “antibody” means not only intact antibody molecules, but also includes fragments thereof which retain antigen binding ability.

When the antibody used in the methods of the invention is a polyclonal antibody (IgG), the antibody is generated by inoculating a suitable animal with a CAPER protein, peptide or a fragment thereof. Antibodies produced in the inoculated animal which specifically bind the CAPER protein are then isolated from fluid obtained from the animal. CAPER antibodies may be generated in this manner in several non-human mammals such as, but not limited to goat, sheep, horse, rabbit, and donkey. Methods for generating polyclonal antibodies are well known in the art and are described, for example in Harlow, et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY). These methods are not repeated herein as they are commonly used in the art of antibody technology.

When the antibody used in the methods of the invention is a monoclonal antibody, the antibody is generated using any well known monoclonal antibody preparation procedures such as those described, for example, in Harlow et al. (supra) and in Tuszynski et al. (1988, Blood, 72:109-115). Given that these methods are well

known in the art, they are not replicated herein. Generally, monoclonal antibodies directed against a desired antigen are generated from mice immunized with the antigen using standard procedures as referenced herein. Monoclonal antibodies directed against full length or peptide fragments of CAPER protein may be prepared
5 using the techniques described in Harlow, et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY).

Techniques for detecting antibody binding are well known in the art. Antibody binding to a CAPER protein or fragment thereof may be detected through the use of chemical reagents that generate a detectable signal that corresponds to the level of
10 antibody binding and, accordingly, to the level of CAPER protein expression. In one of the preferred immunocytochemistry methods of the invention, antibody binding is detected through the use of a secondary antibody that is conjugated to a detectable label. Examples of detectable labels include but are not limited to polymer-enzyme conjugates. The enzymes in these complexes are typically used to catalyze the
15 deposition of a chromogen at the antigen-antibody binding site, thereby resulting in cell staining that corresponds to expression level of the biomarker of interest. Enzymes of particular interest include horseradish peroxidase (HRP) and alkaline phosphatase (AP). Commercial antibody detection systems, such as, for example the
20 Dako Envision+ system (Dako North America, Inc., Carpinteria, CA) and Mach 3 system (Biocare Medical, Walnut Creek, CA), may be used to practice the present invention.

In some aspects of the invention, slides are reviewed microscopically by a cytotechnologist and/or a pathologist to assess cell staining (i.e., CAPER overexpression and/or subcellular distribution as either predominantly cytoplasmic or
25 nuclear). Alternatively, samples may be reviewed via automated microscopy or by personnel with the assistance of computer software that facilitates the identification of positive staining cells.

Detection of antibody binding to CAPER can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various
30 enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include

streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include
5 luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H .

CAPER levels can be detected by aptamer-based assays, which are very similar to antibody-based assays, but with the use of an aptamer instead of an antibody. An aptamer can be any polynucleotide, generally a RNA or a DNA, which
10 has a useful biological activity in terms of biochemical activity or molecular recognition attributes. Usually, an aptamer has a molecular activity such as having an enzymatic activity or binding to a polypeptide at a specific region (i.e., similar to an epitope for an antibody) of the polypeptide or. It is generally known in the art that an aptamer can be made by in vitro selection methods.

In vitro selection methods include a well known method called systematic evolution of ligands by exponential enrichment (a.k.a. SELEX). Briefly, in vitro selection involves screening a pool of random polynucleotides for a particular polynucleotide that binds to a biomolecule, such as a polypeptide, or has a particular activity that is selectable. Generally, the particular polynucleotide represents a very
20 small fraction of the pool, therefore, a round of amplification, usually via polymerase chain reaction, is employed to increase the representation of potentially useful aptamers. Successive rounds of selection and amplification are employed to exponentially increase the abundance of a particular aptamer. In vitro selection is described in Famulok, M.; Szostak, J. W., In Vitro Selection of Specific Ligand
25 Binding Nucleic Acids, *Angew. Chem.* 1992, 104, 1001. (*Angew. Chem. Int. Ed. Engl.* 1992, 31, 979-988.); Famulok, M.; Szostak, J. W., Selection of Functional RNA and DNA Molecules from Randomized Sequences, *Nucleic Acids and Molecular Biology*, Vol 7, F. Eckstein, D. M. J. Lilley, Eds., Springer Verlag, Berlin, 1993, pp. 271; Klug, S.; Famulok, M., All you wanted to know about SELEX; *Mol. Biol. Reports* 1994, 20,
30 97-107; and Burgstaller, P.; Famulok, M. Synthetic ribozymes and the first deoxyribozyme; *Angew. Chem.* 1995, 107, 1303-1306 (*Angew. Chem. Int. Ed. Engl.* 1995, 34, 1189-1192), US Patent No. 6,287,765, US Patent No. 6,180,348, US Patent

No. 6,001,570, US Patent No. 5,861,588, US Patent No. 5,567,588, US Patent No. 5,475,096, and US Patent No. 5,270,163, which are incorporated herein by reference.

B.2. Detection of CAPER RNA

5 In some embodiments, gene expression of CAPER is detected by determining the level of the corresponding mRNA in a breast tissue sample. mRNA expression may be determined by any suitable method. Methods for determining the level of CAPER mRNA expression in a sample are well known in the art and include, but are not limited to, polymerase chain reaction analyses, Northern analyses, and probe
10 arrays. Many expression detection methods use isolated RNA. Any RNA isolation technique that does not select against the isolation of mRNA can be utilized for the purification of RNA from breast tissue samples (see, e.g., Ausubel, ed., 1999, Current Protocols in Molecular Biology (John Wiley & Sons, New York). Additionally, large numbers of tissue samples can readily be processed using techniques well known to
15 those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski, 1989, U.S. Pat. No. 4,843,155). The invention is not limited to any one method of mRNA detection or quantification recited herein, but rather encompasses all presently known or heretofor unknown methods, such as methods that are discovered in the art.

20 In one embodiment, CAPER mRNA is detected in a sample by the process of nucleic acid amplification, e.g., by reverse transcriptase polymerase chain reaction (RT-PCR; the experimental embodiment set forth in Mullis, 1987, U.S. Pat. No. 4,683,202), ligase chain reaction (Barany, 1991, Proc. Natl. Acad. Sci. USA, 88:189 193), self sustained sequence replication (Guatelli, 1990, Proc. Natl. Acad. Sci. USA, 87:1874 1878), transcriptional amplification system (Kwoh, 1989, Proc. Natl. Acad. Sci. USA, 86:1173 1177), Q-Beta Replicase (Lizardi, 1988, Bio/Technology, 6:1197), rolling circle replication (Lizardi, U.S. Pat. No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using
25 techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are
30 present in very low numbers.

In RT-PCR, RNA is enzymatically converted to complementary DNA or "cDNA" using a reverse transcriptase enzyme. The cDNA is then used as a template

for a PCR reaction. PCR products can be detected by any suitable method, including but not limited to, gel electrophoresis and staining with a DNA specific stain or hybridization to a labeled probe. In some embodiments, the quantitative reverse transcriptase PCR with standardized mixtures of competitive templates method
5 described in U.S. Pat. Nos. 5,639,606, 5,643,765, and 5,876,978 (each of which is herein incorporated by reference) is utilized.

CAPER expression levels of RNA may be monitored using a membrane blot (such as used in hybridization analysis such as Northern, dot, and the like), or microwells, sample tubes, gels, beads or fibers (or any solid support comprising
10 bound nucleic acids). See U.S. Pat. Nos. 5,770,722, 5,874,219, 5,744,305, 5,677,195 and 5,445,934, which are incorporated herein by reference. The detection of CAPER expression may also comprise using nucleic acid probes in solution.

The term "Northern blot," as used herein refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size
15 followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled probe to detect RNA species complementary to the probe used. Northern blots are a standard tool of molecular biologists (Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, NY).

The term "probe" refers to any molecule that is capable of selectively binding to a specifically intended target biomolecule, for example, a nucleotide transcript or a protein encoded by or corresponding to CAPER. Probes can be synthesized by one of skill in the art, or derived from appropriate biological preparations. Probes may be specifically designed to be labeled with a detectable label. Examples of molecules that
20 can be used as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic molecules.

One method for the detection of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe can be, for example, a full-length
30 cDNA, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to an mRNA or genomic DNA encoding a biomarker of the

present invention. Hybridization of an mRNA with the probe indicates that CAPER is being expressed.

In one embodiment, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative embodiment, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in an Affymetrix gene chip array (Santa Clara, CA). A skilled artisan can readily adapt known mRNA detection methods for use in detecting the level of mRNA encoding CAPER.

C. Methods

The invention provides a method of determining the progression of a disease state of a subject from DCIS to IDC in a subject afflicted with a breast carcinoma or breast lesion such as DCIS, wherein the levels of CAPER in a breast tissue test sample obtained from the subject are evaluated relative to a control sample. The progression of the disease state of a subject is determined by the progression of the disease state of the breast lesion.

The invention also provides a method of monitoring the progression of a disease state of a subject from DCIS to IDC in a subject afflicted with a breast carcinoma or breast lesion, wherein the levels of CAPER in a breast tissue test sample obtained from the subject are evaluated relative to a control sample. In one embodiment, the control and test samples are taken from the same breast lesion. In another embodiment, the control and test samples are taken from different breast lesions.

In one embodiment, a test sample of breast tissue is obtained from a subject with DCIS. The test sample is obtained from the region of the breast identified as having a palpable or non-palpable breast lesion by standard methods known in the art, such as mammography or ultrasound techniques. CAPER expression is determined in the test sample and compared to CAPER expression levels determined for a control sample. In one embodiment, the control sample is a pooled sample containing breast tissue affected by DCIS obtained from a population of individuals. As described above, when the control sample is obtained from multiple tissue samples, CAPER

expression level can be expressed as an arithmetic mean, median, mode, or other suitable statistical measure of CAPER expression measured in each sample that contributes to the control sample.

In another embodiment, at least two breast tissue samples are obtained from a subject afflicted with a breast carcinoma or a premalignant condition such as DCIS where at least one sample is a control sample and at least one sample is a test sample. In one embodiment of the invention, the control samples and the test sample are obtained from the same breast lesion in one subject. In another embodiment of the invention, the control sample and the test sample are obtained from different breast lesions in one subject. In another embodiment of the invention, the breast tissue samples are obtained at different time points with the sample obtained at the earlier time point acting as the control for test samples obtained at later time points. The level of CAPER in the tissue samples is determined and compared to each other. An elevated CAPER level in the sample taken from the later time point (test sample) relative to the level of CAPER in the sample taken at the earlier (control) time, indicates that the subject has developed IDC.

Samples may be taken over a period of time spanning months or years. For example, tissue samples may be taken every 3 months for up to 3, up to 5, or up to 10 years. It is understood that tissue samples may be taken at a lesser or greater time interval for greater or lesser periods of time.

A breast tissue sample may be prepared according to methods known in the art so that both mRNA levels and protein levels may be assessed in a given sample. The level of CAPER may be expressed in absolute or arbitrary units, as discussed above.

D. Kits

The invention also includes a kit. In one embodiment, a kit of the invention comprises at least one reagent of the invention. A reagent of the invention may comprise an antibody or probe useful in determining the level of CAPER expression in a breast tissue sample. Accordingly, in one embodiment, a kit may comprise a reagent of the invention, buffers or carriers suitable for dissolving or suspending a reagent of the invention and instructional materials. In another embodiment, a kit may further comprise a control sample to be compared to a test sample obtained from a human.

In another embodiment, a kit of the invention comprises materials and instructional material which describe, for instance, a method of determining the progression of a disease state from DCIS to IDC in a human subject by comparing CAPER expression in a test sample obtained from a human subject to CAPER expression in a control sample.

In yet another embodiment, a kit of the invention comprises materials and instructional material which describe, for instance, a method of monitoring the progression of a disease state from DCIS to IDC in a human subject by comparing CAPER expression in a test sample obtained from a human subject to CAPER expression in a control sample.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression, which can be used to communicate the usefulness of the invention in the kit for determining the progression of DCIS to IDC. The instructional material of the kit of the invention may, for example, be affixed to a container, which contains a reagent of the invention or be shipped together with a container, which contains a reagent. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the reagent be used cooperatively by the recipient.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1: CAPER protein expression in human breast cancers

Antibodies

Rabbit polyclonal antibody against CAPER was purchased from Biovision (Mountain View, CA). Monoclonal mouse anti-human antibodies directed against cytokeratin, clones AE1/AE3, were purchased from DAKO (Carpinteria, CA).

Hematoxylin counterstain was purchased from Sigma-Aldrich (St. Louis, MO).

Immunohistochemistry and automated quantitative analyses

Immunohistochemistry and automated quantitative image-based analysis of protein expression within tissue sections (PM2000 AQUA[®] analysis system; Histo Rx, Inc., New Haven, CT) were performed on sections of a tissue array constructed by cutting-edge matrix-assembly (CEMA) (LeBaron et al., 2007, Endocrinol. 148:989-1008). The tissue microarray consisted of 84 invasive ductal carcinoma samples (IDCs) graded as either well- (IDC1), moderately- (IDC2), or poorly-differentiated (IDC3), and 20 normal breast tissue samples.

Sections were subjected to deparaffinization, rehydration and antigen retrieval using citrate buffer, pH 6.0 (DAKO). Subsequently, tissues were blocked with peroxidase blocking reagent (Dako; Cat# S2001) and followed by 10% goat serum (Biogenex; Cat# HK- 112-9K). Sections were then incubated with anti-CAPER IgG, as described above, at 1:100 dilution. Following the incubation, sections were washed 3 times with TBS containing 0.01% Tween-20 (TBST) and then incubated with a mouse anti-cytokeratin antibody (DAKO, Cat# AE1/AE3) for 1 hour. The CAPER antibody was detected using an anti-rabbit HRP-conjugated secondary antibody (DAKO, Envision-Plus), followed by incubation with Tyramide-Cy5 (Perkin Elmer, Cat# NEL745). Cytokeratin was visualized by further incubating the sections with a mouse secondary antibody conjugated to Alexa 488 (Molecular Probes, Cat# A11034). Finally, all sections were stained with DAPI (Vector, Cat: H1500) for nuclear visualization.

Automated quantitative analysis was performed using the AQUA/PM2000 Imaging Platform (HistoRx), as described (Dolled-Filhart et al., 2006, Clin. Cancer Res. 12:6459-6468; LeBaron et al., 2007, Endocrinology 148:989-1008; Wang et al., 2008, Cancer Res. 68 :5628-5638). Briefly, tissue array slides were scanned and images of each breast cancer tissue were captured at different channels including FITC/Alexa 488, Cy5, or DAPI. AQUA software was then used to identify epithelial masks based on FITC-positive cytokeratin-expressing cells. AQUA scores for CAPER represent average signal intensities within epithelial cells.

Statistical Analysis

All the statistical analysis was performed using a Tukey-Kramer Multiple Comparison test. A p value of < 0.05 was considered significant.

Results

Quantitative analysis of CAPER expression in a human breast cancer tissue microarray was achieved by using the PM2000 Automated Quantitative Analysis (AQUA) system (HistoRx, Inc., New Haven, CT). CAPER protein expression is significantly elevated in all three tumor grades (IDC1 = well-differentiated, IDC2 = moderately-differentiated, and IDC3 = poorly-differentiated; Figure 1A-C).

Normal breast tissue (Normal) and breast tissue afflicted with various stages of invasive ductal carcinoma (IDC) were assayed for CAPER and Cytokeratin protein expression using immunohistochemistry (Figure 1A). Cytokeratin is used to identify normal and neoplastic tissue of epithelial origin. CAPER expression is elevated in tissue from IDC1, IDC2, and IDC3, but most highly over-expressed in moderately-differentiated breast cancers (i.e. IDC2).

CAPER protein expression in the tissue array samples was quantitated using the AQUA[®] automated image acquisition and analysis system. The distribution and mean AQUA[®] scores for CAPER protein expression in normal and IDC tissue using PM2000 AQUA automated quantitative analysis system are depicted in Figure 1B. Relative to normal breast tissue samples (Normal), breast tissue afflicted with well differentiated IDC (1), tissue afflicted with moderately differentiated IDC (2), and tissue afflicted with poorly differentiated IDC (3) all had elevated CAPER protein expression. Confirming the immunohistochemistry results, CAPER protein is most highly over-expressed in moderately-differentiated breast cancers. Mean AQUA scores for CAPER protein expression in normal and IDC tissue are depicted in Figure 1C. Breast tissue samples afflicted with IDC1, IDC2, or IDC3 all had significantly elevated CAPER expression relative to normal control tissue samples.

Example 2: Enhanced CAPER mRNA expression distinguishes Invasive Ductal Carcinoma (IDC) from Ductal Carcinoma in situ (DCIS)

Methods

Forty-eight tissue samples were obtained from patients afflicted with either DCIS or IDC (Stage 1-4). Tissue samples were identified and combined according to disease stage as either DCIS, IDC1, IDC2, IDC3, or IDC4. At least five- to ten independent patient tissue samples were combined for each pooled sample.

First strand cDNA was prepared from RNA isolated from fresh and frozen human samples. Quantitative real-time PCR was performed using primers that selectively amplified human CAPER cDNA. The sequences for real time PCR to detect CAPER were as follows:

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Forward:	GTGGCTCTTTGGCATTGTTGGT	SEQ ID NO. 3
Reverse:	GCTGGGAAAGTCTTGTTTGCA	SEQ ID NO. 4

Relative quantification of samples was normalized by arbitrarily setting the average value for DCIS to 1. Changes in CAPER transcript levels in IDC samples were then expressed as a multiple thereof (i.e. relative expression). The differences in the number of mRNA copies in each PCR reaction was corrected using mouse GAPDH endogenous control transcript levels. Error bars represent standard deviation.

Results

Relative expression of CAPER mRNA across IDC samples obtained from subjects with Stage 1, stage 2, stage 3, or stage 4 IDC were not significantly different from each other (Figure 2; $p < 0.05$). However, CAPER mRNA expression is relatively upregulated up to 2.5-fold in all breast tissue samples afflicted with IDC relative to breast tissue samples afflicted with DCIS.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed:

1. A method for determining the progression of a disease state from
5 Ductal carcinoma in situ (DCIS) to Invasive Ductal Carcinoma (IDC) in a human
subject afflicted with a breast lesion, said method comprising:

(1) obtaining at least one test sample of breast tissue from the subject,
wherein said test sample is obtained from the region of the lesion;

(2) determining the level of CAPER expression in said test sample; and

10 (3) comparing the level of CAPER expression determined in said test
sample to the level of CAPER expression determined in a DCIS control sample,
wherein an elevated level of CAPER expression in said test sample
relative to the level of CAPER expression in said control sample indicates that the
disease state of the subject has progressed from DCIS to IDC.

15 2. The method of claim 1, wherein said control sample is obtained
from the same subject as the test sample.

20 3. The method of claim 1, wherein said control sample comprises
pooled samples containing breast tissue samples obtained from a population of
individuals.

4. The method of claim 1, wherein said human subject is a woman.

25 5. The method of claim 1, wherein said method of determining the
level of CAPER expression comprises an assay for CAPER mRNA.

6. The method of claim 5, wherein the assay is selected from the group
consisting of a Northern Blot analysis, in situ hybridization, and RT-PCR.

30 7. The method of claim 5, wherein the level of CAPER mRNA in said
sample is elevated at least 2-fold relative to the level of CAPER mRNA in the control.

8. The method of claim 1, wherein said method of determining the level of CAPER expression comprises an assay for CAPER protein.

9. The method of claim 8, wherein said assay is selected from the group consisting of Western blot analysis, radioimmunoassay (RIA), and immunoassay, chemiluminescent assay, or enzyme-linked immunosorbent assay (ELISA).

10. A method of monitoring the progression of a disease state from Ductal carcinoma in situ (DCIS) to Invasive Ductal Carcinoma (IDC) in a human subject afflicted with a breast lesion, said method comprising:

(1) obtaining at least one first sample of breast tissue afflicted with DCIS from a human subject at a first time point and at least one second sample of breast tissue afflicted with a breast lesion from a second time point;

(2) determining the levels of CAPER expression in said first and second samples, and

(3) comparing the level of CAPER expression determined in said first and second samples, wherein an elevated level of CAPER expression in said second sample relative to said first sample indicates that the disease state of the subject has progressed from DCIS to IDC.

11. The method of claim 10, wherein the level of CAPER expression determined in said second sample is compared to a DCIS control sample, wherein an elevated level of CAPER expression in said second sample relative to said control sample indicates that the disease state of the subject has progressed from DCIS to IDC.

12. The method of claim 11, wherein said control sample comprises pooled samples containing breast tissue samples obtained from a population of individuals, where said samples are afflicted with DCIS.

13. The method of claim 10, wherein said human subject is a woman.

14. The method of claim 10, wherein said method of determining the level of CAPER expression comprises an assay for CAPER mRNA.

5 15. The method of claim 14, wherein the assay is selected from the group consisting of a Northern Blot analysis, in situ hybridization, and RT-PCR.

16. The method of claim 14, wherein the level of CAPER mRNA in said sample is elevated at least 2-fold relative to the level of CAPER mRNA in the control.

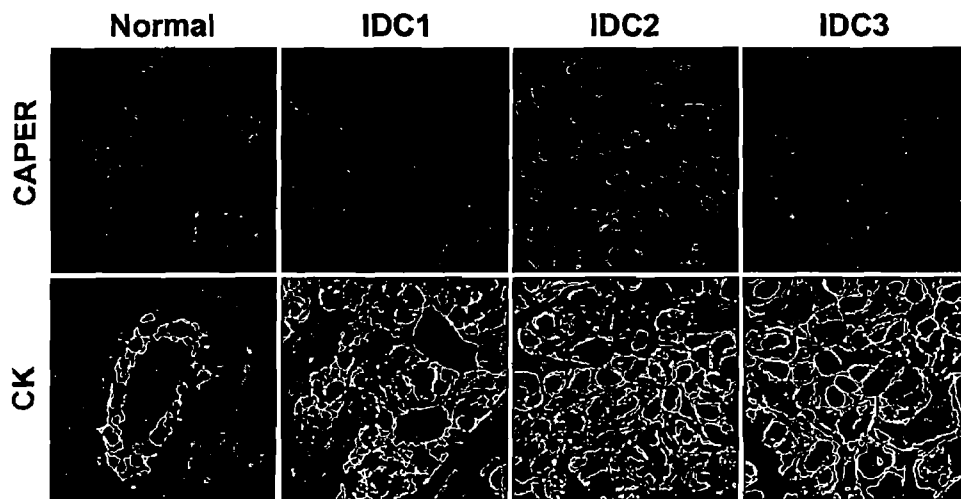
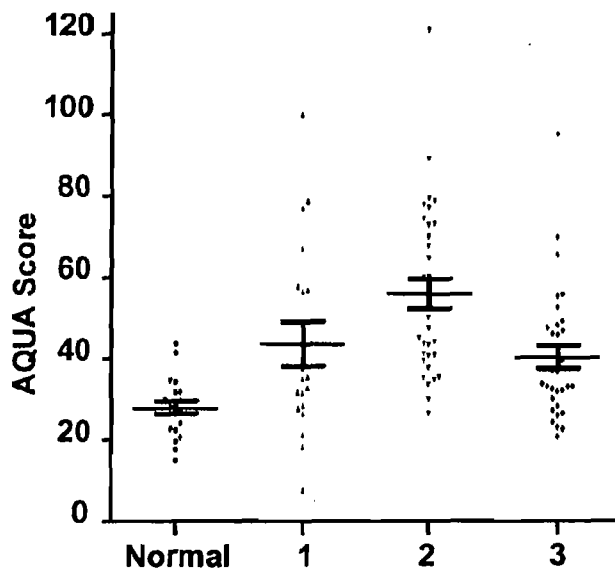
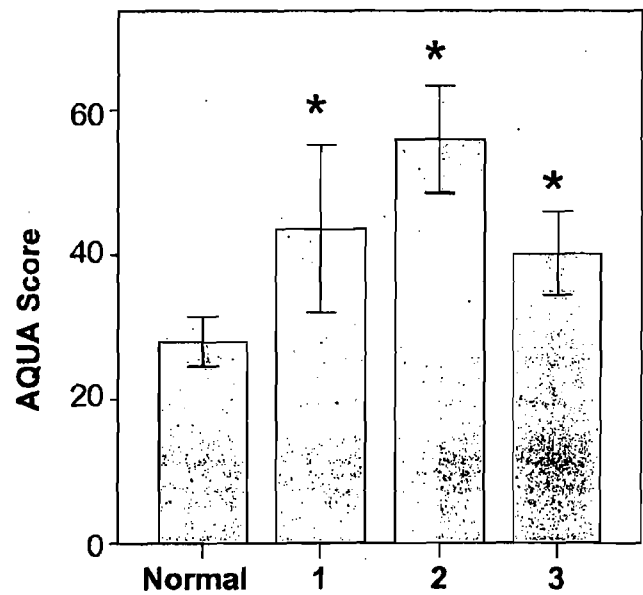
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17. The method of claim 10, wherein said method of determining the level of CAPER expression comprises an assay for CAPER protein.

15 18. The method of claim 17, wherein said assay is selected from the group consisting of Western blot analysis, radioimmunoassay (RIA), and immunoassay, chemiluminescent assay, or enzyme-linked immunosorbent assay (ELISA).

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Figure 1**A****B****C**

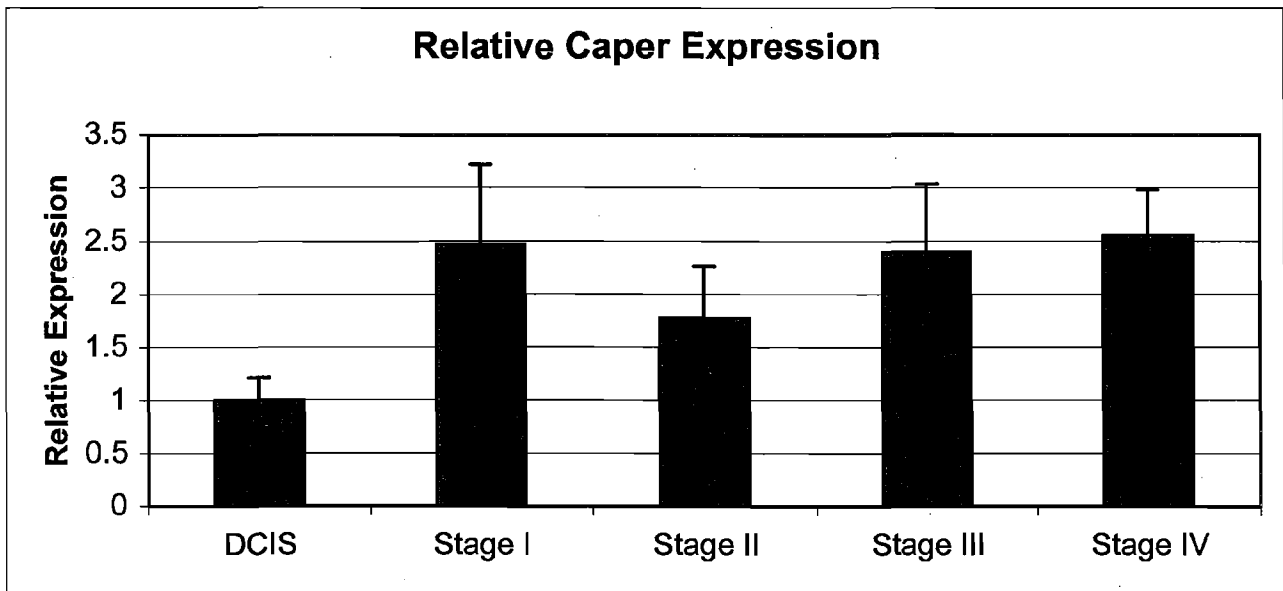


Figure 2.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/65510

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/48 (2010.01)

USPC - 436/64, 63, 86

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC - 436/64, 63, 86

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

USPC - 436/501

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST(DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=YES; OP=ADJ), Google Scholar(Michael P. LISANTI CAPER, Isabelle MERCIER CAPER, Jean-Francois JASMIN CAPER, Hallgeir RUI CAPER, Thai Hong TRAN CAPER, Richard G. PESTELL CAPER, Ductal carcinoma CAPER, Ductal carcinoma RBM39, Ductal carcinoma RBB23, Ductal carcinoma CG11266)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2004/002911 A1 (MACK ET AL.), 12 February 2004 (12.02.2004); para [0014], [0211], [0217]; pg 65 Table 4, PKey# 131320, CC1.3	1-18
Y	US 6,964,868 B1 (WILLIAMS ET AL.), 15 November 2005 (15.11.2005); col 42, ln 64-66; col 43, ln 6 - 18; col 44, ln 9-17; col 45, ln 43-52, 56-59; col 625; Table 2, SEQ ID NO: 4835	1-18

☐ Further documents are listed in the continuation of Box C.


* Special categories of cited documents:

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

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

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

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

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

"T"

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

"X"

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

"Y"

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

"&"

document member of the same patent family

Date of the actual completion of the international search

2 February 2010 (02.02.2010)

Date of mailing of the international search report

25 FEB 2010

Name and mailing address of the ISA/US

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