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(54) Title: METHODS OF PROGNOSING EARLY STAGE BREAST LESIONS

Fig. 1 A
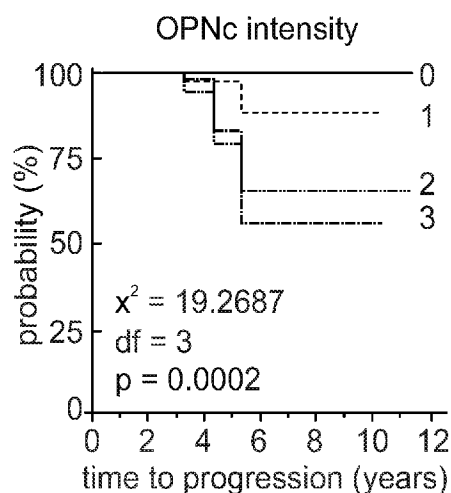
(57) Abstract: A method is provided for prognosing a risk in a patient diagnosed with a premalignant lesion of the breast of the lesion progressing to breast cancer, the method comprising: providing a tissue sample of the premalignant lesion; detecting one or more variants of Osteopontin (OPN) selected from OPN-a, OPN-b and OPN-c in the sample; and prognosing an elevated risk of the lesion progressing to breast cancer if OPN-b and/or OPN-c is detected and/or OPN-a is elevated above normal levels in the tissue sample. Methods of assessing risk of death from breast cancer, methods of treatment, and kits for prognosis are also provided herein.

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METHODS OF PROGNOSING EARLY STAGE BREAST LESIONS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001]   This application claims priority to U.S. Provisional Application No. 62/554,712 filed September 6, 2017, the contents of which are incorporated herein by reference.

FIELD

[0002]   The invention relates to methods of determining or predicting breast cancer using biomarkers expressed or absent in premalignant lesions.

BACKGROUND

[0003]   Women that are found to have preinvasive lesions of the breast historically are faced with only three options following their diagnosis: observation; chemoprevention; or surgery (e.g., lumpectomy or mastectomy). There are, unfortunately, no predictors for progression risk that are specific to the woman's diagnosis to guide the decision regarding which path to follow. Distinguishing high-risk patients from low-risk patients would improve the prognosis of high-risk patients (through early decisive intervention) and spare unnecessary treatment for the low-risk patients (through continued observation).

[0004]   Currently available diagnostic techniques in breast cancer prevention involve a biopsy, where samples of tissue are taken to confirm or eliminate the presence of transformed cells by histopathological examination. These processes lack indicators for the detection of invasive potential, may understate the disease identified, and the early stages of breast transformation (atypias) are difficult to differentiate from benign growths (hyperplasias) on one end of the spectrum and invasive carcinomas in situ (CIS) on the other end. Furthermore, the inspection of tumor margins to assess invasiveness is

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unreliable and it requires step sections through the entire biopsy material. Microinvasion is typically identified at surgical biopsy, as core needle tissue cannot enable this distinction.

[0005]   Hence, there is an intense need for dependable biomarkers. The lack of reliable molecular indicators for breast cancer progression, however, has led to efforts to use increasingly complex readouts. Multiple tumor markers, including HER2 amplification/over-expression, cathepsin D, and uPAR, have been considered for prognostication and therapy decisions of breast cancer in a Tumor Marker Utility Grading System (Hayes et al., *Breast Cancer Res. Treat.* 52:305-19, 1998). A gene prognosis profile of 70 genes, developed at the Netherlands Cancer Institute, has been characterized as a good predictor of outcome (van de Vijver et al., *N. Engl. J. Med.* 347:1999-2009, 2002). The likelihood of distant recurrence in breast cancer patients, who have estrogen receptor positive tumors and no involved lymph nodes, can be defined with a panel of 21 gene products, amplified by RT-PCR from paraffin blocks, and correlated with the likelihood of distant recurrence (Paik et al., *N. Engl. J. Med.* 351:2817-2826, 2004).

[0006]   As such, there exists a need for improved molecular biomarker assays and processes for prognosing breast tumor invasiveness at an early stage of diagnosis such as through effective analyses of premalignant breast lesions.

                                    SUMMARY

[0007]   The following summary of the invention is provided to facilitate an understanding of some of the innovative features unique to the present invention and is not intended to be a full description. A full appreciation of the various aspects of the invention can be gained by taking the entire specification, claims, drawings, and abstract as a whole.

[0008]   In one embodiment, a method is provided for prognosing a risk in a patient diagnosed with a premalignant lesion of the breast, of the lesion progressing to breast

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cancer, the method comprising: providing a tissue sample of the premalignant lesion; detecting one or more variants of Osteopontin (OPN) selected from OPN-a, OPN-b and OPN-c in the sample; and prognosing an elevated risk of the lesion progressing to breast cancer if OPN-b and/or OPN-c is detected and/or OPN-a is elevated above normal levels in the tissue sample.

[0009] In another embodiment, a method is provided of assessing risk of death from breast cancer in a patient diagnosed with a premalignant lesion of the breast, the method comprising: providing a sample of the lesion and immunohistochemically detecting OPN-a, OPN-b and/or OPN-c in the sample by detecting selective anti-OPN-c and anti-OPN-a/b stains; and assessing the risk of death as elevated if OPN-c is detected.

[0010] In another embodiment, a method is provided of treating a patient diagnosed with a premalignant lesion of the breast, the method comprising: prognosing a risk of the lesion progressing to breast cancer by providing a tissue specimen of the premalignant lesion, detecting one or more variants of Osteopontin (OPN) selected from OPN-a, OPN-b and OPN-c in the sample, and prognosing an elevated risk of the lesion progressing to breast cancer if OPN-c is detected in the tissue sample; and treating the patient by administering chemoprevention therapy or surgery if an elevated risk is prognosed, or subjecting the patient to continued observation without chemoprevention or surgery if an elevated risk is not prognosed.

[0011] In a further embodiment, a kit for the prognosis of premalignant breast cancer lesions is provided, the kit comprising: an antibody selected for OPN-c; an antibody selective for OPN exon 4; and reagents for conducing an immunohistochemical analysis of a sample of a premalignant breast cancer lesion.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 illustrates OPN splice variants are indicators for prognosis. Kaplan-Meier curves for the risk of progression over time. The x-axis shows the time of follow-up in years, the y-axis displays the probability of remaining recurrence-free cases in

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percent of the total number. The measured variables in A,C are categorical. The variables in B,D are dichotomized. The χ^2 statistic is inserted into the lower left corner of the graphs, df = degrees of freedom. **A)** OPN-c intensity. **B)** OPN-c intensity dichotomized. **C)** OPN exon 4 intensity. **D)** OPN exon 4 intensity dichotomized.

5   **[0013]**   FIG. 2 illustrates that pathology scores prognosticate outcome subgroups. **A,C)** Shown are the distributions of pathology scores for marker staining intensity (0-3 as indicated to the left of the bars, with 0 = unfilled, 1 = dotted, 2 = hatched, 3 = filled) for 139 patients who do or do not succumb to cancer (A) and 140 patients who do or do not experience progression (C) over a time frame of 5 years (5+ indicates that some

10   patients remained free of adverse outcome over more than 5 years, shorter follow-ups are disregarded). The left two bars reflect LF161 staining (OPN exon 4), whereas the right two bars indicate anti-OPN-c staining. The y-axis shows the number of patients as percent of the total in each group. **B,D)** To utilize the information of both markers and to gain discrimination, the pathology scores for OPN-c and OPN exon 4 marker intensities

15   were combined and their distributions visualized on stacked bar graphs (low = pathology scores for both markers 0-1, intermediate = one marker 0-1 the other 2-3, high = both markers 2-3, as indicated to the left of the bars; low = unfilled, intermediate = hatched, high = filled) for patients who do or do not succumb to cancer (B) and patients who do or do not experience ensuing invasive disease (D) over a time frame of 5 years. The y-axis

20   shows the number of patients as percent of the total in each group.

[0014]   FIG. 3: illustrates that ROC curves validate the OPN splice variants as markers for progression and survival. Shown are graphs for survival **(A)** and progression **(B)**. All indicators are above the diagonal. Evaluated are the pathology scores for OPN-c staining intensity alone, OPN exon 4 staining intensity alone, both markers combined,

25   and a logistic regression analysis including all of the variables under study. The non-smooth curve-fit for the combined analysis reflects the iterations of the logistic

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regression as calculated in R. int = staining intensity, combined = sum of pathology scores for OPN-c and OPN exon 4 staining.

[0015]   FIG. 4: illustrates survival, reflected in Kaplan-Meier Curves, prognosticated by OPN-c staining.   Survival curves in relation to OPN-c staining intensity for all patients with follow-up information (left panel; N = 42, 122), DCIS patients (middle panel; N = 19, 82), and patients with premalignant lesions other than DCIS (right panel; N = 23, 40).   The change in Y-axis scale is intended to highlight the difference between OPN-c low (intensity score 0-1) and OPN-c high (intensity score 2-3), even though the risk for recurrence differs among the subgroups of patients.

[0016]   FIG. 5: illustrates multivariate analysis for the combination of prognostic indicators.   A) Evaluated were the pathology scores for OPN exon 4 and OPN-c together with risk group for prognosticating recurrence. The graph represents a Kaplan-Meier curve for the risk of recurrence over time. The x-axis shows the time of follow-up in years, the y-axis displays the probability of remaining recurrence-free cases. B) Kaplan-Meier curves for the risk of recurrence over time by diagnosis. The x-axis shows the time of follow-up in years, the y-axis displays the probability of remaining progression-free cases. The measured variables are categorical. The χ^2 statistic is inserted into the lower left corner of the graph, df = degrees of freedom. In both graphs, the scale of the y-axis is stretched to maximize resolution.

[0017]   FIG. 6: illustrates DCIS outcome under treatment. Distributions of combined pathology scores for OPN-c and OPN exon 4 marker intensities (open box = low = pathology scores for both markers 0-1, hatched box = intermediate = one marker 0-1 the other 2-3, filled box = high = both markers 2-3) for patients who do or do not experience invasive disease over a time frame of 5 years. A) The distribution of combined pathology scores differs only moderately among treatment groups (tamoxifen + radiation n=41, radiation n=39, tamoxifen n=12). B) The distribution of combined pathology scores differs only moderately between tamoxifen-treated and not tamoxifen-treated patients (tamoxifen n=53, not treated n=62). C and D) Among treated patients, the combined

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pathology score may predict risk, but very low numbers require confirmation in a larger patient population. **C)** Tamoxifen and/or radiation (non-progression n=59, progression n=3). **D)** Tamoxifen (non-progression n=4, progression n=3).

SEQUENCE LISTING

[0018]   Applicant hereby incorporates by reference a CRF sequence listing submitted herewith having a file name 10738-652_Sequence_Listing.txt created on September 5, 2018.

[0019]   The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

[0020]   SEQ ID NO: 1 represents a peptide sequence of OPN-a.

[0021]   SEQ ID NO: 2 represents a peptide sequence of OPN-b.

[0022]   SEQ ID NO: 3 represents a peptide sequence of OPN-c.

[0023]   SEQ ID NO: 4 represents a coding nucleic acid sequence of OPN-a.

[0024]   SEQ ID NO: 5 represents a coding nucleic acid sequence of OPN-b.

[0025]   SEQ ID NO: 6 represents a coding nucleic acid sequence of OPN-c.

[0026]   SEQ ID NO: 7 represents an epitope found specifically in OPN-c.

[0027]   SEQ ID NO: 8 represents an epitope found specifically in OPN-a and OPN-b.

DETAILED DESCRIPTION

[0028]   The following description of particular embodiment(s) is merely exemplary in nature and is in no way intended to limit the scope of the invention, its application, or uses, which may, of course, vary. The invention is described with relation to the non-limiting definitions and terminology included herein. These definitions and terminology

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are not designed to function as a limitation on the scope or practice of the invention but are presented for illustrative and descriptive purposes only.

[0029]   The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms, including "at least one," unless the content clearly indicates otherwise. "Or" means "and/or." As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. It will be further understood that the terms "comprises" and/or "comprising," or "includes" and/or "including" when used in this specification, specify the presence of stated features, regions, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, regions, integers, steps, operations, elements, components, and/or groups thereof. The term "or a combination thereof" means a combination including at least one of the foregoing elements.

[0030]   Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. It will be further understood that terms such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and the present disclosure, and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

[0031]   The processes and kits as described herein have utility as a predictor of elevated risk of a premalignant lesion of the breast progressing to breast cancer in a subject. As such, provided are processes of prognosing a risk of a premalignant lesion of the breast in a patient progressing to breast cancer. The invention identifies and categorizes expression of one or more of the gene products of cytokine Osteopontin (OPN, Spp1). OPN in broad sense has been extensively studied as a metastasis gene representing the most abundantly secreted phospho-protein in breast and other cancers

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and is believed to support invasive behavior. As such, OPN is a biomarker for breast cancer aggressiveness and for breast cancer prognosis where the abundance of Osteopontin correlates negatively with survival. In older studies, pan-Osteopontin (total Osteopontin, typically covering all variant forms) was measured. (Weber GF, et al., *Brit J Cancer* 2010; 103:861-869, Weber GF, et al., *Oncol Reports* 2011; 25:433-441) It was found that pan-Osteopontin correlated with premalignant progression in breast and other transformations (Weber GF, et al., *Brit J Cancer* 2010; 103:861-869).

[0032]   The gene product of OPN, however, is subject to alternative splicing selectively in cancer, which deletes exon 4 (27 amino acids) to generate Osteopontin-c (OPN-c), or exon 5 (to generate Osteopontin-b (OPN-b)) from the unspliced form (called Osteopontin-a (OPN-a)). OPN-c lacks exon 4 in the NH_2-terminal region of the mature sequence. OPN-c lacks the transglutaminase reactive domain (Gly-X-Gly) which can mediate covalent homodimer cross-linking as well as heterodimer formation to other matrix components (such as fibronectin). This description provides processes that examine the levels by intensity or percent positivity of histological samples of premalignant lesions of the breast to determine whether or prognosticate if the lesions will become breast cancer in the subject.

[0033]   Processes as provided herein include prognosing risk of progressing to breast cancer in a subject or assessing risk of death in a subject as a relation between the extent of expression of one of more OPN variants alone or optionally in conjunction with a measure of pathology score, detection intensity, percent positivity, or risk category. Processes in some aspects as provided herein include providing a tissue sample of a premalignant breast lesion from a subject, detecting one or more variants of Osteopontin (OPN) in the sample where the variants are OPN-a, OPN-b or OPN-c, and prognosing an elevated risk of the lesion progressing to breast cancer if OPN-b and/or OPN-c is detected and/or OPN-a is elevated above normal levels in the tissue sample. It was found specifically, that elevated risk of the lesion progressing to breast cancer was observed particularly with levels of OPN-c that could be categorized as high independent of the

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type of lesion found in the subject. In addition, the presence of high levels OPN-b/a expression, particularly in high risk lesions correlated with increased chance of breast cancer in subjects.

[0034]   As used herein a "subject" is a mammal. Optionally, a subject is a human or non-human primate. Optionally, a subject is a dog, cat, equine, sheep, bovine, rabbit, pig, or murine.

[0035]   As used herein, the term "tissue sample" is defined as sample obtained from a biological organism, a tissue, cell, or any medium suitable for mimicking biological conditions, or from the environment. Illustratively, a tissue sample includes breast tissue, such as that obtained during a needle biopsy, lumpectomy, or mastectomy. Samples, such as tissue samples, can be placed on microscope slides. In particular examples, samples are used directly, or can be manipulated prior to use, for example, by fixing (e.g., using formalin) or embedding (e.g., in plastic or paraffin). A sample is optionally a nucleic sample meaning that the sample includes material or is specifically selected to be derived from or target a nucleus. Optionally, a sample is a cytoplasmic sample meaning that the sample includes or is specifically selected to be derived from or target cytoplasm.

[0036]   The term "breast cancer" as used herein includes any tumor of the breast, such as tumors of epithelial (carcinoma) or stromal (sarcoma) breast tissue. Exemplary *in situ* epithelial breast cancers include ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). LCIS is a tumor that consists of abnormal cells in the lining of a lobule. DCIS is made up of abnormal cells in the lining of a duct. Exemplary invasive breast carcinomas include carcinoma NOS (not otherwise specified), lobular carcinoma, tubular/cribriform carcinoma, mucinous (colloid) carcinoma, medullary carcinoma, papillary carcinoma, and metaplastic carcinoma. An exemplary breast sarcoma is phyllodes tumor.

[0037]   A process as provided herein includes detecting the presence or absence of one or more variants of OPN. A variant of OPN may be OPN-a, OPN-b or OPN-c. The

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amino acid sequences of the OPN variants are known. An exemplary sequence of OPN-a in a human is MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLL APQNAVSSEETNDFKQETLPSKSNESHDHMDDMDDEDDDDHVDSQDSIDSNDS

5   DDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDS VVYGLRSKSKKFRRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSD WDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKANDESNEHSDVIDSQELSKVS REFHSHEFHSHEDMLVVDPKSKEEDKHLKFRISHELDSASSEVN (SEQ ID NO: 1). An OPN-a sequence includes a wild-type (or native) sequence of SEQ ID NO: 1, as well

10   as OPN-a variants (e.g., polymorphisms) expressed in breast cancer cells. In certain examples, OPN-a has at least 80% sequence identity, for example at least 85%, at least 90%, at least 95%, or at least 98% sequence identity to SEQ ID NO: 1.

[0038]   An   exemplary   sequence   of   OPN-b   in   a   human   is MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLL

15   APQTLPSKSNESHDHMDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDE SHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRR PDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQL DDQSAETHSHKQSRLYKRKANDESNEHSDVIDSQELSKVSREFHSHEFHSHEDM LVVDPKSKEEDKHLKFRISHELDSASSEVN (SEQ ID NO: 2). An OPN-b sequence

20   includes a wild-type (or native) sequence of SEQ ID NO: 2, as well as OPN-b variants (e.g., polymorphisms) expressed in breast cancer cells. In certain examples, OPN-b has at least 80% sequence identity, for example at least 85%, at least 90%, at least 95%, or at least 98% sequence identity to SEQ ID NO: 2.

[0039]   An   exemplary   sequence   of   OPN-c   in   a   human   is

25   MRIAVICFCLLGITCAIPVKQADSGSSEEKQNAVSSEETNDFKQETLPSKSNESHD HMDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDF PTDLPATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRRPDIQYPDATDEDITS HMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSR

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LYKRKANDESNEHSDVIDSQELSKVSREFHSHEFHSHEDMLVVDPKSKEEDKHL

KFRISHELDSASSEVN (SEQ ID NO: 3). An OPN-c sequence includes a wild-type (or

native) sequence of SEQ ID NO: 3, as well as OPN-c variants (e.g., polymorphisms)

expressed in breast cancer cells. In certain examples, OPN-c has at least 80% sequence

5   identity, for example at least 85%, at least 90%, at least 95%, or at least 98% sequence

identity to SEQ ID NO: 3.

[0040]   An exemplary coding nucleic acid sequence of human OPN-a is

ctccctgtgttggtggaggatgtctgcagcagcatttaaattctgggagggcttggttgtcagcagcagcaggaggaggcagag

cacagcatcgtcgggaccagactcgtctcaggccagttgcagccttctcagccaaacgccgaccaaggaaaactcactaccat

10   gagaattgcagtgatttgcttttgcctcctaggcatcacctgtgccataccagttaaacaggctgattctggaagttctgaggaaaa

gcagctttacaacaaatacccagatgctgtggccacatggctaaaccctgacccatctcagaagcagaatctcctagccccaca

gaatgctgtgtcctctgaagaaaccaatgactttaaacaagagacccttccaagtaagtccaacgaaagccatgaccacatggat

gatatggatgatgaagatgatgatgaccatgtggacagccaggactccattgactcgaacgactctgatgatgtagatgacactg

atgattctcaccagtctgatgagtctcaccattctgatgaatctgatgaactggtcactgattttcccacggacctgccagcaaccg

15   aagttttcactccagttgtccccacagtagacacatatgatggccgaggtgatagtgtggtttatggactgaggtcaaaatctaag

aagtttcgcagacctgacatccagtaccctgatgctacagacgaggacatcacctcacacatggaaagcgaggagttgaatggt

gcatacaaggccatccccgttgcccaggacctgaacgcgccttctgattgggacagccgtgggaaggacagttatgaaacga

gtcagctggatgaccagagtgctgaaacccacagccacaagcagtccagattatataagcggaaagccaatgatgagagcaa

tgagcattccgatgtgattgatagtcaggaactttccaaagtcagccgtgaattccacagccatgaatttcacagccatgaagatat

20   gctggttgtagaccccaaaagtaaggaagaagataaacacctgaaatttcgtatttctcatgaattagatagtgcatcttctgaggt

caattaaaaggagaaaaaatacaatttctcactttgcatttagtcaaaagaaaaaatgctttatagcaaaatgaaagagaacatgaa

atgcttctttctcagtttattggttgaatgtgtatctatttgagtctggaaataactaatgtgtttgataattagtttagtttgtggcttcatg

gaaactccctgtaaactaaaagcttcagggttatgtctatgttcattctatagaagaaatgcaaactatcactgtattttaatatttgtta

ttctctcatgaatagaaatttatgtagaagcaaacaaaatacttttacccacttaaaaagagaatataacattttatgtcactataatctt

25   ttgttttttaagttagtgtatattttgttgtgattatcttttgtggtgtgaataaatctttatcttgaatgtaataagaatttggtggtgtcaa

ttgcttatttgtttccacggttgtccagcaattaataaaacataacctttttactgcctaaaaaaaaaaaaaaaa (SEQ ID

NO: 4).

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[0041]   An exemplary coding nucleic acid sequence of human OPN-b is

ctccctgtgttggtggaggatgtctgcagcagcatttaaattctgggagggcttggttgtcagcagcagcaggaggaggcagag

cacagcatcgtcgggaccagactcgtctcaggccagttgcagccttctcagccaaacgccgaccaaggaaaactcactaccat

gagaattgcagtgatttgcttttgcctcctaggcatcacctgtgccataccagttaaacaggctgattctggaagttctgaggaaaa

gcagctttacaacaaatacccagatgctgtggccacatggctaaaccctgacccatctcagaagcagaatctcctagccccaca

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ggactccattgactcgaacgactctgatgatgtagatgacactgatgattctcaccagtctgatgagtctcaccattctgatgaatct

gatgaactggtcactgattttcccacggacctgccagcaaccgaagttttcactccagttgtccccacagtagacacatatgatgg

ccgaggtgatagtgtggtttatggactgaggtcaaaatctaagaagtttcgcagacctgacatccagtaccctgatgctacagac

gaggacatcacctcacacatggaaagcgaggagttgaatggtgcatacaaggccatccccgttgcccaggacctgaacgcgc

cttctgattgggacagccgtgggaaggacagttatgaaacgagtcagctggatgaccagagtgctgaaacccacagccacaa

gcagtccagattatataagcggaaagccaatgatgagagcaatgagcattccgatgtgattgatagtcaggaactttccaaagtc

agccgtgaattccacagccatgaatttcacagccatgaagatatgctggttgtagaccccaaaagtaaggaagaagataaacac

ctgaaatttcgtatttctcatgaattagatagtgcatcttctgaggtcaattaaaaggagaaaaaatacaatttctcactttgcatttagt

caaaagaaaaaatgctttatagcaaaatgaaagagaacatgaaatgcttctttctcagtttattggttgaatgtgtatctatttgagtct

ggaaataactaatgtgtttgataattagtttagtttgtggcttcatggaaactccctgtaaactaaaagcttcagggttatgtctatgttc

attctatagaagaaatgcaaactatcactgtattttaatatttgttattctctcatgaatagaaatttatgtagaagcaaacaaaatacttt

tacccacttaaaaagagaatataacatttttatgtcactataatcttttgtttttttaagttagtgtatattttgttgtgattatctttttgtggtgt

gaataaatctttatcttgaatgtaataagaatttggtggtgtcaattgcttatttgtttttcccacggttgtccagcaattaataaaacata

accttttttactgcctaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa (SEQ ID NO: 5).

[0042]   An exemplary coding nucleic acid sequence of human OPN-c is

ctccctgtgttggtggaggatgtctgcagcagcatttaaattctgggagggcttggttgtcagcagcagcaggaggaggcagag

cacagcatcgtcgggaccagactcgtctcaggccagttgcagccttctcagccaaacgccgaccaaggaaaactcactaccat

gagaattgcagtgatttgcttttgcctcctaggcatcacctgtgccataccagttaaacaggctgattctggaagttctgaggaaaa

gcagaatgctgtgtcctctgaagaaaccaatgactttaaacaagagacccttccaagtaagtccaacgaaagccatgaccacat

ggatgatatggatgatgaagatgatgatgaccatgtggacagccaggactccattgactcgaacgactctgatgatgtagatgac

actgatgattctcaccagtctgatgagtctcaccattctgatgaatctgatgaactggtcactgattttcccacggacctgccagca

accgaagttttcactccagttgtccccacagtagacacatatgatggccgaggtgatagtgtggtttatggactgaggtcaaaatc

13

taagaagtttcgcagacctgacatccagtaccctgatgctacagacgaggacatcacctcacacatggaaagcgaggagttga

atggtgcatacaaggccatccccgttgcccaggacctgaacgcgccttctgattgggacagccgtgggaaggacagttatgaa

acgagtcagctggatgaccagagtgctgaaacccacagccacaagcagtccagattatataagcggaaagccaatgatgaga

gcaatgagcattccgatgtgattgatagtcaggaactttccaaagtcagccgtgaattccacagccatgaatttcacagccatgaa

gatatgctggttgtagaccccaaaagtaaggaagaagataaacacctgaaatttcgtatttctcatgaattagatagtgcatcttctg

aggtcaattaaaaggagaaaaaatacaatttctcactttgcatttagtcaaaagaaaaaatgctttatagcaaaatgaaagagaac

atgaaatgcttctttctcagtttattggttgaatgtgtatctatttgagtctggaaataactaatgtgtttgataattagtttagtttgtggct

tcatggaaactccctgtaaactaaaagcttcagggttatgtctatgttcattctatagaagaaatgcaaactatcactgtattttaatatt

tgttattctctcatgaatagaaatttatgtagaagcaaacaaaatacttttacccacttaaaaagagaatataacattttatgtcactata

atctttttgtttttttaagttagtgtatattttgttgtgattatctttttgtggtgtgaataaatcttttatcttgaatgtaataagaatttggtggtg

tcaattgcttatttgttttcccacggttgtccagcaattaataaaacataacctttttttactgcctaaaaaaaaaaaaaaaaaa (SEQ

ID NO: 6).

[0043] A process optionally is able to detect the expression of, expression level of (e.g. staining intensity), or positivity of one or more of OPN-a, OPN-b, and OPN-c either by histochemical staining or other suitable technique. In some aspects, immunohistochemistry (IHC) is used to detect one or more OPN variants.

[0044] Immunohistochemistry utilizes antibodies to selectively bind to a target of interest, and more particularly to an epitope present on a target of interest. The term "selectively binds" refers, with respect to an antigen such as an OPN variant, to the preferential association of an antibody or other specific binding agent, in whole or part, to the antigen and not to other antigens. Selective binding results in a relatively strong association between the binding agent (e.g., antibody) and the antigen than between the antibody and a protein that does not express an epitope for the antibody.

[0045] The binding agent is optionally coupled with a detection agent such as a fluorophore, nuclide, enzyme (e.g. horse radish peroxidase), biotin or avidin, or other suitable molecule that is capable of emitting a signal directly or following coupling with or reaction with a secondary molecule. The detection agent is an agent capable of detection, for example by spectrophotometry, flow cytometry, or microscopy. For

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example, one or more binding agents can be attached to an antibody, thereby permitting detection of the target protein. Optionally, one or more labels can be attached to a nucleic acid probe, thereby permitting detection of the target nucleic acid molecule. Exemplary labels include radioactive isotopes, fluorophores, ligands, chemiluminescent agents, enzymes, and combinations thereof.

[0046]   The binding agent is optionally an antibody that is selective for one or more variants of OPN. Optionally, an antibody is selective for OPN-a and does not significantly cross react with either OPN-b or OPN-c or any other molecule within a sample. Optionally, an antibody is selective for OPN-b and does not significantly cross react with either OPN-a or OPN-c or any other molecule within a sample. Optionally, an antibody is selective for OPN-c and does not significantly cross react with either OPN-a or OPN-b or any other molecule within a sample. An illustrative example of an antibody that is selective for OPN-c is one that selectively binds the epitope SEEKQNAVS (SEQ ID NO: 7) or a variant thereof. Illustrative examples of such antibodies include AhOPNc that is an IgY that may be obtained from Gallus Immunotech or Exalpha Biologicals, Inc., Shirley, MA.

[0047]   In some aspects, an antibody is cross reactive between two or more OPN variants and as such may selectively detect both the presence or absence of 2 variants. Optionally, an antibody cross reacts with OPN-a and OPN-b (OPN-a/b), OPN-a and OPN-c (OPN-a/c), or OPN-b and OPN-c (OPN-b/c). Optionally an antibody is directed to OPN-a/b by recognition of an epitope selectively found on OPN-a and OPN-b that has the sequence LYNKYPDAVATWLNPDPSQKQNLLAPN (SEQ ID NO: 8). An illustrative example of an antibody that is cross reactive between OPN-a and OPN-b is LF161 available from Larry Fisher (National Institute of Dental and Craniofacial Research/NIH) or Kerafast (Boston, MA).

[0048]   An antibody is optionally polyclonal or monoclonal. One of ordinary skill in the art understands how to produce antibodies by standard techniques and screen the resulting monoclonal or polyclonal antibodies for their ability to interact with an epitope

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sequence. Such methods are illustratively taught by Monoclonal Antibodies: Methods and Protocols, Albitar, M, ed., Humana Press, 2010 (ISBN 1617376469); and Antibodies: A Laboratory Manual, Harlos, E, and Lane, D. eds., Cold Spring Harbor Laboratory Press, 1988 (ISBN-10: 0879693142). Optionally, an antibody includes or is

5   IgG, IgA, IgM, or other antibody type. A naturally occurring antibody (such as IgG, IgM, IgD) and those that result from inoculation of a mammal for production of antibodies, includes four polypeptide chains, two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. As used herein, the term "antibody" also includes recombinant antibodies produced by expression of a nucleic acid that encodes one or

10   more antibody chains in a cell (for example see U.S. Pat. No. 4,745,055; U.S. Pat. No. 4,444,487; WO 88/03565; EP 256,654; EP 120,694; EP 125,023; Faoulkner et al., *Nature* 298:286, 1982; Morrison, *J. Immunol.* 123:793, 1979; Morrison et al., *Ann Rev. Immunol* 2:239, 1984).

       [0049]   The term antibody also includes an antigen binding fragment of a naturally

15   occurring or recombinant antibody. Specific, non-limiting examples of binding fragments encompassed within the term antibody include Fab, $(Fab')_2$, Fv, and single-chain Fv (scFv) as each of these terms are recognized in the art. Methods of making these fragments are routine in the art.

       [0050]   The presence of the detection agent on the antibody or other specific binding

20   agent allows a sample to be analyzed for intensity of the interaction of the OPC variant in the sample. Intensity is recognizes as the magnitude (absolute or relative) of the signal observed following selective binding to the OPC variant in the sample. Intensity is optionally detected independently for nuclei and cytoplasm. Optionally, when IHC is used the scale of detectable staining intensity is represented on a typical 1 to 3 scale to

25   represent the intensity, wherein 0 is assigned to negative staining, 3 being assigned to very intensely staining samples, and 1 assigned to weakly staining samples. In some examples a value of 1 or 2 may be re-tested. One skilled in the art will appreciate that

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these values are not absolutes and the magnitude of physical staining intensity may vary depending on the samples and reagents used.

[0051]   In some embodiments a process includes determining the percent positivity of a sample for the presence of one or more OPC variants. Percent positivity is optionally defined as the percentage of cells in the field or sample that show a positive result for one or more target OPC variants.

[0052]   Optionally, a pathology score is assigned to each sample. A pathology score is a singular score that includes the value for intensity (e.g. 0, 1, 2, or 3), the percent positivity (on a scale of 0, 1, 2, or 3), or a combination thereof. Thus, a pathology score represents the relative value assigned to either the intensity or the percent positivity. As such one or more pathology scores are optionally included in a process.

[0053]   In some embodiments a pathology score may be dichotimized into values that may be considered low (denoted L) or high (denoted H). For example, for intensity a pathology score of 0 or 1 may be categorized as L. A pathology score of 2 or 3 may be categorized as H. Similarly, for percent positivity a pathology score of 0 or 1 may be categorized as L and a pathology score of 2 or 3 may be categorized as H. Optionally, a lesion is an intermediate risk category lesion where intermediate risk is defined as one of either intensity or percent positivity being L and the other of intensity or percent positivity being H.

[0054]   In some aspects, a lesion is assigned a risk category that may be based on the pathology score. For example, sine neoplasmate and ductal hyperplasia lesions may be placed in a low risk category. Flat epithelial atypia (FEA), papillomatosis intraductalis (PI), atypical ductal hyperplasia (ADH) and lobular carcinoma in situ (LCIS) may be placed into an intermediate risk category. Finally, ductal carcinoma in situ (DCIS) may be placed in a high risk category.

[0055]   In some aspects, a logistic regression may be used to determine a relative risk for breast cancer in the subject based on the detection of pathology score, intensity, percent positivity, or risk category. For logical regression it is possible to use one or

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more of the foregoing or all four. Optionally, a ROC (receiver operating characteristic) curve can be generated as a graphical plot of sensitivity/specificity as the discrimination threshold is varied. To model outcome (survival or progression) one may use logistic regression using Formula I:

$$\pi(X_1, X_2, X_3, X_4, X_5) = \frac{e^{\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5}}{1 + e^{\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5}}$$

(Formula I)

with X indicating the parameters measured (4 pathology scores as categorical variables and risk as a dichotomized variable) and β being the coefficient for the regression (calculated in R, a language and environment for statistical computing and graphics). The log odds are a linear function of the covariates.

[0056]   Prognosing the risk of progressing to breast cancer or assessing the risk of death may be categorized as low or elevated. Optionally, the low or elevated is a relative value and dependent on the result of the pathology score and the risk category as well as either dependent on whether the pathology score is derived from the levels of intensity and/or percent positivity for OPN-a, OPN-b, OPN-c, or a combination of any of the foregoing, optionally OPN-a/b. It was found that the pathology score of OPN-c, independently when used with risk category, correlates with risk of progressing to breast cancer or risk of death such that when a sample exhibits an H for pathology score and the lesion is assigned to a high, intermediate, or low risk category, the risk of progressing to breast cancer may be prognosed as elevated. Similarly, when the pathology score in a sample is H for OPN-a/b and the lesion is assigned to a high risk category, the risk of progressing to breast cancer is prognosed as elevated. With respect to risk of death the presence of the presence of detectable OPN-c optionally independent of whether the OPN-c is of high or low intensity, of high or low percent positivity, or the use of risk category of a lesion used in the process. In other aspects a risk of death is assessed as elevated when OPN-a/b is determined to have a pathology score of H alone or in

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conjunction with the use of measurements specific of the intensity, percent positivity, or the use of risk category of a lesion used in the process.

[0057]   Optionally, a subject is administered a treatment. A treatment is optionally correlated to a determined elevated risk of progressing to breast cancer or risk of death according to the processes as provided herein. A treatment is optionally an invasive or otherwise aggressive treatment or is a non-aggressive treatment. A non-aggressive treatment optionally includes monitoring for a predetermined or indefinite period of time. Monitoring is optionally for 1 or more years, optionally 2 or more years, optionally 5 or more years, or optionally 10 or more years. Monitoring is optionally once or more times per year. Monitoring optionally includes imaging such as MRI, mammogram, or other known monitoring method, blood screening, or other monitoring method.

[0058]   In some aspects a risk of death or risk of progressing to breast cancer is considered elevated. In such circumstances a subject may be administered an intermediate or aggressive treatment. An aggressive treatment is optionally a mastectomy, a lumpectomy combined with radiotherapy and/or chemotherapy (e.g., anti-HER2, anti-ER, and/or anti-PR therapies for example Trastuzumab (Herceptin®), bevacizumab (Avastin®), pertuzumab (OmniTarg™), ZM105180 (Zemab®), ertumaxomab (Rexonum), Arimidex®, raloxifene, and tamoxifen), or an increase in the dose and/or number of courses of chemotherapy. Methods of administering such therapies are routine in the art and can be designed by skilled clinicians.

[0059]   In some aspects an intermediate therapy protocol is administered. Illustrative examples of an intermediate therapy include a lumpectomy instead of a mastectomy, a lumpectomy alone instead of a lumpectomy combined with radiotherapy and/or chemotherapy, or a decrease in the dose and/or number of courses of chemotherapy. Methods of administering such therapies are routine in the art and can be designed by skilled clinicians.

[0060]   Also provided are kits that may be used in one or more of the processes as provided herein. A kit optionally includes an antibody selective for OPN-c or otherwise

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capable of selectively binding to SEQ ID NO: 7; an antibody selective for OPN exon 4 (optionally that suitable for specifically binding to SEQ ID NO: 8); and one or more reagents for conducting an immunohistochemical analysis of a sample of a premalignant breast cancer lesion. One or more reagents for conducting immunohistochemical analysis

5   of a sample of a premalignant breast cancer lesion optionally include antibodies, nucleic acids, or other reagents suitable for detection and optionally quantifying expression of an OPN variant. The reagent is optionally an antibody that is selective for one or more variants of OPN. Optionally, an antibody is selective for OPN-a and not significantly cross reacting with either OPN-b or OPN-c or any other molecule within a sample.

10   Optionally, an antibody is selective for OPN-b and not significantly cross reacting with either OPN-a or OPN-c or any other molecule within a sample. Optionally, an antibody is selective for OPN-c and not significantly cross reacting with either OPN-a or OPN-b or any other molecule within a sample. An illustrative example of an antibody that is selective for OPN-c is one that selectively binds SEQ ID NO: 7 or a variant thereof.

15   Illustrative examples of such antibodies include AhOPNc that is an IgY that may be obtained from Gallus Immunotech or Exalpha Biologicals, Inc., Shirley, MA.

[0061]   In some aspects, an antibody used in a kit as provided herein is cross reactive between two or more OPN variants and as such may selectively detect both the presence or absence of 2 variants. Optionally, an antibody cross reacts with OPN-a and OPN-b

20   (OPN-a/b), OPN-a and OPN-c (OPN-a/c), or OPN-b and OPN-c (OPN-b/c). Optionally an antibody as provided in a kit is directed to OPN-a/b by recognition of an epitope of SEQ ID NO: 8 or a variant thereof. An illustrative example of an antibody that is cross reactive between OPN-a and OPN-b is LF161 available from Larry Fisher (National Institute of Dental and Craniofacial Research/NIH) or Kerafast (Boston, MA).

25   Antibodies may be any other suitable antibody that may be prepared as described herein or as otherwise known in the art.

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[0062]   Specific processes, compositions and kits as described herein are illustrated in the following examples. These examples are provided for illustrative purposes and are not considered limitations on the scope of inventions.

<div align="center">EXPERIMENTAL</div>

[0063]   **Subjects:** Biopsies from a total of 434 women with premalignant breast lesions comprising 343 patients from Wroclaw, Poland and 91 patients from Cincinnati, USA are assessed for expression of OPN-a/b or OPN-c and used for the prognostication of developing breast cancer or risk of death based on expression, pathology score alone and/or in conjunction with risk category of the lesion. The diagnoses of the lesions tested from subjects ranged from healthy breast tissue or usual ductal hyperplasia via atypia/atypical ductal hyperplasia or papillary breast lesions or LCIS to DCIS. All DCIS patients had a resection, 12 were treated with tamoxifen alone, 39 with radiation alone, and 41 with tamoxifen plus radiation therapy. The patients in Poland were followed up to 5 years, the patients in the US initially presented 2005-2011. The Cincinnati patients were not sequential specimens. Because the incidence of invasion after DCIS or atypia is very low, they were selected in two groups, those who subsequently developed invasive cancer and those who did not. The lead investigator and biostatisticians were blinded to this selection. The study was approved by the ethics committees at Wroclaw Medical University, Poland and the University of Cincinnati, USA.

[0064]   Of 434 patients, 54 women had healthy breast tissue (sine neoplasmate) and 60 had usual ductal hyperplasia. These two groups are considered low risk for progression. Atypia/ADH (74 cases), papilloma/papillomatosis intraductalis (19 cases) and LCIS (20 cases) comprise an intermediate progression risk (risk of subsequent invasive cancer increases from 1.5–2.0% for proliferative lesions without atypia, to 3.5–5.0% for hyperplastic lesions with atypia (Page et al. 1985)). The 198 patients with DCIS (ductal carcinoma in situ) have an elevated risk to develop breast cancer. The risk level of radial scar is not fully characterized; 9 patients with this diagnosis were included in

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the overall evaluation without assignment to risk groups. Follow-up information was available for a fraction of the subjects as indicated below.

[0065]   **Immunohistochemistry:** Antibodies AhOPNc or LF161 were commercially obtained and used for immunohistochemical analyses of the tissue samples. For each antibody, a formalin-fixed and paraffin-embedded biopsy specimen from premalignant lesions was cut on a microtome in 5 μm slices. Antibodies were used after blocking samples in 2% donkey serum. Antibodies were diluted 1:500 to 1:700. The polyclonal rabbit antibody LF161 for staining selectively exon 4 (present in Osteopontin-a and -b) was used at 1:1000. The antibodies and their use in immunohistochemistry have been thoroughly validated (Zduniak et al. 2015 and references therein Shen/Weber 2014; Zduniak et al. 2016). For each antibody, the tissues were scored according to intensity (maximum intensity of the sample 0, 1, 2, or 3) and percent positivity (0, 1, 2, or 3), separately for nuclei and cytoplasm. In addition to analyzing the indicators in their original scale, immunohistochemical biomarkers were dichotomized into low (0–1) or high (2–3). This method was determined to strengthen the power of the analysis (Zduniak et al. 2016). All microscopic slides were independently evaluated by two pathologists, and in the rare cases of discrepant initial scores, a final score was assigned after discussion.

[0066]   The anti-Osteopontin exon-4 antibody, which recognizes OPN-a and -b, stained selectively the cytoplasm. Lesions displayed OPN-c predominantly in their nuclei (lesion-free breasts had no staining). The markers (OPN-c nuclear intensity, OPN-c nuclear percent positivity, exon 4 cytoplasmic intensity, exon 4 cytoplasmic percent positivity) showed increases in average pathology scores with higher transformation risk (from low via intermediate to high). OPN-c was more stringently associated with the elevated risk groups than exon 4, reaching significant p-values for staining intensity as well as for percent positivity in all comparisons. Further, for each subgroup comparison,

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OPN-c staining intensity and percent positivity, but not OPN exon 4 staining, reached significant levels of difference between diagnostic entities (Table 1).

Table 1: Pathology Scores in Distinct Subgroups Correlate with Risk

			OPNa/b		OPNc	
			intensity	percent	intensity	percent
low risk	intermediate risk	χ^2	9.635	5.065	68.385	57.794
		P	0.022	0.167	<.0001	<.0001
low risk	elevated risk	χ^2	27.600	27.703	129.889	134.821
		P	<.0001	<.0001	<.0001	<.0001
intermediate risk	elevated risk	χ^2	18.753	16.021	14.868	15.635
		P	0.000	0.001	0.002	0.001
SN	ADH	χ^2	8.313	12.704	72.489	62.720
		P	0.040	0.005	<.0001	<.0001
UDH	ADH	χ^2	8.151	1.752	19.261	10.382
		P	0.043	0.625	0.000	0.016
SN	DCIS	χ^2	48.796	49.294	176.546	176.087
		P	<.0001	<.0001	<.0001	<.0001
UDH	DCIS	χ^2	5.018	4.977	49.278	54.982
		P	0.171	0.174	<.0001	<.0001
ADH	DCIS	χ^2	15.007	14.304	15.401	16.015
		P	0.002	0.003	0.002	0.001

[0067] Table 1 shows χ^2 test for differences in pathology scores (staining intensity followed by percent positivity) among various premalignant diagnoses. P = p-value (underlined if lower than 0.05). The upper portion of the Table shows the evaluation of the main risk groups; low risk comprises SN (sine neoplasmate) and UDH (usual ductal hyperplasia); intermediate risk entails atypia/ADH (atypical ductal hyperplasia), papilloma/papillomatosis, LCIS (lobular carcinoma in situ); elevated risk is DCIS (ductal carcinoma in situ). The lower section compares pairwise the diagnostic subgroups with the largest patient numbers. OPNa/b denotes staining for exon 4, OPNc denotes staining for the splice junction of OPN-c.

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[0068]   **Statistics:**   Statistical analyses were conducted using MedCalc version 14.8.1. The pathology scores assess staining intensity and percent positivity. The predictors were each categorical or dichotomized (pathology scores 0 and 1 = low versus 2 and 3 = high). A second analysis included the risk group. For evaluating differences in biomarkers among the risk groups (obtained from pathology scores and the premalignant diagnoses) a χ^2 test was applied. The primary methods for addressing survival time (duration) and prognosis (ensuing invasive disease or death) was Kaplan Meier for univariate analysis. A multivariate analysis of those factors with a p-value of less than 0.05 were then applied to a Cox proportional hazard model. The hazard ratio (HR) measures the hazard between two individuals, whose value of the independent variable differ by one unit (if continuous) or moving from one class to another class (for categorical variable).

[0069]   **Logistic Regression:**   For biomarker development, ROC curves were devised using all parameters (intensity and percent positivity of the immunohistochemistry stains plus risk groups). To model outcome (survival or progression) the following formula was employed:

$$\pi(X_1, X_2, X_3, X_4, X_5) = \frac{e^{\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5}}{1 + e^{\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5}}$$

with X indicating the parameters measured (4 pathology scores as categorical variables and risk as a dichotomized variable) and β being the coefficient for the regression (calculated in R, a language and environment for statistical computing and graphics). The log odds are a linear function of the covariates.

[0070]   **Prognosis:**   Follow-up information had 214 patients with non-recurrence over various observation periods (111 were free of relapse for at least 5 years following the initial diagnosis) and 55 patients (20%) experiencing breast cancer over 3-5 years (48 patients had insufficient follow-up duration or died from other causes and were

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excluded). The data identified OPN-c intensity scores 2-3 as stronger predictors for progression than intensity scores 0-1 for all types of lesions analyzed (Figure 4). For OPN exon 4, the probability of progression increased with score, and moderate gain was achieved by dichotomizing (Figure 1). The dichotomized scores were used for biomarker

5   development. Multi-variate analysis confirmed that the two biomarkers OPN-c and OPN exon 4 are prognostic for ensuing invasive disease, whereas the risk group did not add significantly to the prognostication (consistent with reports that OPN-c is a progression marker for all types of breast cancer (Mirza et al. 2008)). Among the risk groups, expectedly, DCIS was associated with the highest probability of developing breast

10   cancer compared to PI, ADH, and LCIS (Figure 5). A Cox proportional hazards regression model was applied for the variables under consideration. OPN-c intensity had a p-value of 0.0022 and a hazard ratio of 1.8181 (95% confidence limits 1.2427-2.6597). OPN-a/b intensity had a p-value of 0.0220 and a hazard ratio of 1.4456 (95% confidence limits 1.0564-1.9783). By contrast, the values for risk were p-value 0.7185, hazard ratio

15   0.9472 (95% confidence limits 0.7064-1.2702). This suggests that the OPN variant forms are biomarkers for progression hazard, also for lesions that are conventionally categorized as low risk. The markers may be of particular benefit in assessing the need for treatment in non-DCIS premalignant lesions.

[0071]   **Biomarker Properties:** Patients who died from breast cancer within 5 years

20   were evaluated in comparison to those who were alive for at least 5 years following the initial diagnosis. Analysis for the association of outcome with the markers under investigation (OPN-c, OPN exon 4) reflected them as prognostic. The pathology scores were higher for OPN exon 4 as well as for OPN-c in patients who succumbed to breast cancer compared to those who over at least 5 years did not. When combining OPN-c and

25   OPN exon 4 staining intensity on a scale of low (pathology scores for both markers 0-1), intermediate (one marker 0-1 the other 2-3) and high (both markers 2-3), the prognostic accuracy improved such that all of the low patients were alive after 5 years, whereas women in the high category had a 30% chance to die within 5 years (with almost 20% of

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the survivors among them having experienced documented invasive disease). Close to 80% of patients who succumbed had a high score at the time of initial diagnosis (Figure 2A,B). In the intermediate group, a high score for OPN-c was more unfavorable (ratio alive:dead = 6.5:1) than a high score for exon 4 (ratio alive:dead = 15:1).

[0072]   Patients who incurred breast cancer within 5 years were evaluated in comparison to those who were free of relapse for at least 5 years following the initial diagnosis. More than 90% of women who experienced breast cancer had pathology scores of 2-3 for OPN-c intensity at the time of initial diagnosis. About 2.5% of women free of OPN-c (intensity pathology score 0), and 7.5% of OPN-c pathology score 1 progress over 5 years. This risk increases to 24% at pathology score 2 and 40% at pathology score 3. However, OPN exon 4 was less informative than OPN-c (13% at intensity score 0, 21% at score 1, 25% at score 2, 31% at score 3), so that combining the two markers yielded modest improvement over OPN-c intensity alone (Figure 2C,D).

[0073]   According to ROC curves, a logistic regression algorithm that applies the pathology scores as categorical variables and the dichotomized risk group (low or medium versus high) achieves better sensitivity and specificity for the prognostication of death from breast cancer (Figure 3A) as well as for the prognostication of cancer development (Figure 3B) than any of the individual pathology scores alone. The combined information derived from OPN-c staining, OPN exon 4 staining, and diagnosis can provide a foundation for very reliable prognostication.

[0074]   **DCIS Treatment:** All DCIS cases underwent surgical resection (16 patients had mastectomies). A fraction was further treated with tamoxifen (12), radiation (39) or both (41). While the sizes of the subgroups preclude conclusive assessments due to lack of statistical power, the trends suggest that the prognostic value of OPN-c/exon 4 is insignificantly affected by ensuing treatment, and that within each treatment group (either tamoxifen plus radiation or tamoxifen alone) the biomarker may be able to distinguish high versus low risk for invasive disease (Figure 6).

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[0075] **Discussion:** Results identified OPN splice variant -c as a prognostic indicator for ensuing invasive disease and survival following premalignant breast lesions. OPN exon 4 and the diagnosis of the lesion are contributing markers. The observation is consistent with existing knowledge regarding the biological effects of the splice variants.

5 Although the spliced OPN forms are always expressed together with the full-length form OPN-a, their ratios vary (the rate of RNA splicing is different from, and functionally independent of the rate of transcription). While OPN-a and OPN-c may synergize in tumor progression (Weber, et al., Osteopontin is a marker for cancer aggressiveness and patient survival. *Brit J Cancer* **103**:861-869 (2010), OPN-c is more potent in promoting

10 aggressive behavior (He, et al., An osteopontin splice variant induces anchorage independence in human breast cancer. *Oncogene* **25**:2192-2202 (2006)). OPN splice variants have been found to be of value for breast cancer diagnosis/prognosis/prediction. Adding measurements of OPN-c and OPN exon 4 to existing diagnostic workups of precancerous lesions holds promise for assessing invasive potential and for

15 prognosticating cancer risk, which existing markers cannot do.

[0076] Methods involving conventional biological techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 3rd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.,

20 2001; Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates); and Short Protocols in Molecular Biology, ed. Ausubel et al., 52 ed., Wiley-Interscience, New York, 2002. Immunological methods (e.g., preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, e.g., in Current Protocols in

25 Immunology, ed. Coligan et al., John Wiley & Sons, New York, 1991; and Methods of Immunological Analysis, ed. Masseyeff et al., John Wiley & Sons, New York, 1992.

[0077] Methods of producing and screening antibodies are illustratively found in Monoclonal Antibodies: Methods and Protocols, Albitar, M, ed., Humana Press, 2010

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(ISBN 1617376469); and Antibodies: A Laboratory Manual, Harlos, E, and Lane, D. eds., Cold Spring Harbor Laboratory Press, 1988 (ISBN-10: 0879693142).

[0078] Additional protocols such as PCR Protocols can be found in A Guide to Methods and Applications Academic Press, NY. Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel, et al. (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification," Methods in Enzymology vol. 182, and other volumes in this series; Current Protocols in Protein Science, John Wiley and Sons, New York, NY; and manufacturer's literature on use of protein purification products known to those of skill in the art.

[0079] Various modifications of the present invention, in addition to those shown and described herein, will be apparent to those skilled in the art of the above description. Such modifications are also intended to fall within the scope of the appended claims.

[0080] It is appreciated that all reagents are obtainable by sources known in the art unless otherwise specified. Methods of nucleotide amplification, cell transfection, and protein expression and purification are similarly within the level of skill in the art.

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U.S. Patent Application Publication 2018/0119232

[0081]   Patents, applications, and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are incorporated herein by reference to the same extent as if each individual application or publication was specifically and individually incorporated herein by reference.

[0082]   The foregoing description is illustrative of particular embodiments of the invention, but is not meant to be a limitation upon the practice thereof. The following claims, including all equivalents thereof, are intended to define the scope of the invention.

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CLAIMS

1. A method for prognosing a risk in a patient diagnosed with a premalignant lesion of the breast, of the lesion progressing to breast cancer, the method comprising:

    providing a tissue sample of the premalignant lesion;

    detecting one or more variants of Osteopontin (OPN) selected from OPN-a, OPN-b and OPN-c in the sample; and

    prognosing an elevated risk of the lesion progressing to breast cancer if OPN-b and/or OPN-c is detected and/or OPN-a is elevated above normal levels in the tissue sample.

2. The method according to claim 1, wherein detecting comprises immunohistochemically staining a sample of the tissue specimen, the sample selected from a nucleic sample, a cytoplasmic sample, or both, and measuring one or more of stain intensity and percent positivity for each stain.

3. The method according to claim 2, wherein immunohistochemically staining comprises contacting the sample with at least one antibody selective for one or more variants of OPN.

4. The method according to claim 3, wherein at least one antibody selectively stains OPN-c (anti-OPN-c), and at least one antibody selectively stains OPN-a and/or OPN-b (anti-OPN-a/b).

5. The method according to claim 4, wherein the antibody selective for OPN-c selectively binds an epitope comprising the sequence SEEKQNAVS or a variant thereof, or the antibody selectively bind an epitope comprising the sequence

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LYNKYPDAVATWLNPDPSQKQNLLAPN, optionally the antibody comprises IgY AhOPNc or LF161.

6.   The method according to any one of claims 1-4, further comprising assigning a pathology score to each sample, the pathology score comprising 0, 1, 2 or 3 for intensity, or 0, 1, 2 or 3 for percent positivity.

7.   The method according to claim 6, further comprising dichotomizing the pathology score into low (L = 0 or 1) or high (H = 2 or 3).

8.   The method according to claim 7, further comprising assigning the lesion to a risk category based on the pathology score, such that sine neoplasmate and ductal hyperplasia lesions comprise a low risk category, flat epithelial atypia (FEA), papillomatosis intraductalis (PI), atypical ductal hyperplasia (ADH) and lobular carcinoma in situ (LCIS) comprise an intermediate risk category, and ductal carcinoma in situ (DCIS) comprise a high risk category.

9.   The method according to claim 8, wherein the premalignant lesion of the breast comprises a low or intermediate risk category lesion.

10.   The method according to claim 8, wherein if the sample exhibits H an anti-OPN-c stain and the lesion is assigned to a high, intermediate or low risk category, the risk of progressing to breast cancer is prognosed as elevated.

11.   The method according to claim 8, wherein if the sample exhibits H anti-OPN-a/b stains and the lesion is assigned to a high risk category, the risk of progressing to breast cancer is prognosed as elevated.

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12. The method according to claim 1 wherein the step of prognosing comprises subjecting a staining intensity or percent positivity of an immunohistochemistry stain for OPN-a/b and OPN-c, and risk group to a logistic regression to thereby determine risk for breast cancer or for death from breast cancer.

13. A method of assessing risk of death from breast cancer in a patient diagnosed with a premalignant lesion of the breast, the method comprising: providing a sample of the lesion and immunohistochemically detecting OPN-a, OPN-b and/or OPN-c in the sample by detecting selective anti-OPN-c and anti-OPN-a/b stains; and assessing the risk of death as elevated if OPN-c is detected.

14. The method of assessing risk of death from breast cancer according to claim 13, further comprising scoring the stains 0, 1, 2 or 3 for intensity and 0, 1, 2, or 3 for percent positivity and classifying the pathology score for each stain as high (H = 2 or 3) or low (L = 0 or 1), or intermediate (I = one L and one H); and assessing the risk of death as elevated if OPN-a/b is detected with a pathology score of H.

15. The method of assessing risk of death from breast cancer according to claim 13, further comprising scoring the stains 0, 1, 2 or 3 for intensity and 0, 1, 2, or 3 for percent positivity and classifying the pathology score for each stain as high (H = 2 or 3) or low (L = 0 or 1), or intermediate (I = one L and one H); and assessing the risk of death as elevated if OPN-c is detected with a pathology score of H.

16. The method of assessing risk of death from breast cancer according to any one of claims 13-15, further comprising assigning the lesion to a risk category based lesion type, such that sine neoplasmate and ductal hyperplasia lesions comprise a low risk category, flat epithelial atypia (FEA), papillomatosis intraductalis (PI), atypical ductal hyperplasia (ADH) and lobular carcinoma in situ (LCIS) comprise

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an intermediate risk category, and ductal carcinoma in situ (DCIS) comprise a high risk category; and assessing risk as elevated if (a) OPN-c is detected with a pathology score of H or I and a lesion risk category of high or intermediate or low, or (b) OPN-a/b is detected with a pathology score of H and a lesion risk category of high or intermediate, or (c) OPN-a/b is detected with a pathology score of H or I and a lesion risk category of H.

17.   A method of treating a patient diagnosed with a premalignant lesion of the breast, the method comprising:

      prognosing a risk of the lesion progressing to breast cancer by providing a tissue specimen of the premalignant lesion, detecting one or more variants of Osteopontin (OPN) selected from OPN-a, OPN-b and OPN-c in the sample, and prognosing an elevated risk of the lesion progressing to breast cancer if OPN-c is detected in the tissue sample; and

      treating the patient by administering chemoprevention therapy or surgery if an elevated risk is prognosed, or

      subjecting the patient to continued observation without chemoprevention or surgery if an elevated risk is not prognosed.

18.   A method of treating a patient according to claim 17, wherein chemoprevention therapy is selected from one or both of administering at least one estrogen receptor modulator and radiation.

19.   The method of treating according to claim 18, wherein the estrogen receptor modulator is selected from raloxifene and tamoxifen.

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20.   A method of treating a patient diagnosed with a premalignant lesion of the breast, the method comprising:

prognosing a risk of the lesion progressing to breast cancer according to any of claims 1 – 4;

administering chemoprevention therapy or surgery to the patient if

(a) the sample exhibits an anti-OPN-c H pathology score and the lesion risk category is high, intermediate or low, or

(b) the sample exhibits anti-OPN-a/b H pathology scores and the lesion risk category is high; and

subjecting the patient to continued observation without chemoprevention therapy or surgery otherwise.

21.   The method of treating according to claim 20, wherein chemoprevention therapy comprises one or both of administering at least one estrogen receptor modulator and optionally administering radiation.

22.   The method according to claim 21, wherein the estrogen receptor modulator is selected from raloxifene and tamoxifen.

23.   A kit for the prognosis of premalignant breast cancer lesions, the kit comprising:

an antibody selective for OPN-c;

an antibody selective for OPN exon 4; and

reagents for conducting an immunohistochemical analysis of a sample of a premalignant breast cancer lesion.

24.   The kit according to claim 21, wherein the antibody selective for OPN-c comprises polyclonal antibodies.

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25.   The kit according to claim 21, wherein the antibody selective for OPN-c comprises a monoclonal antibody.
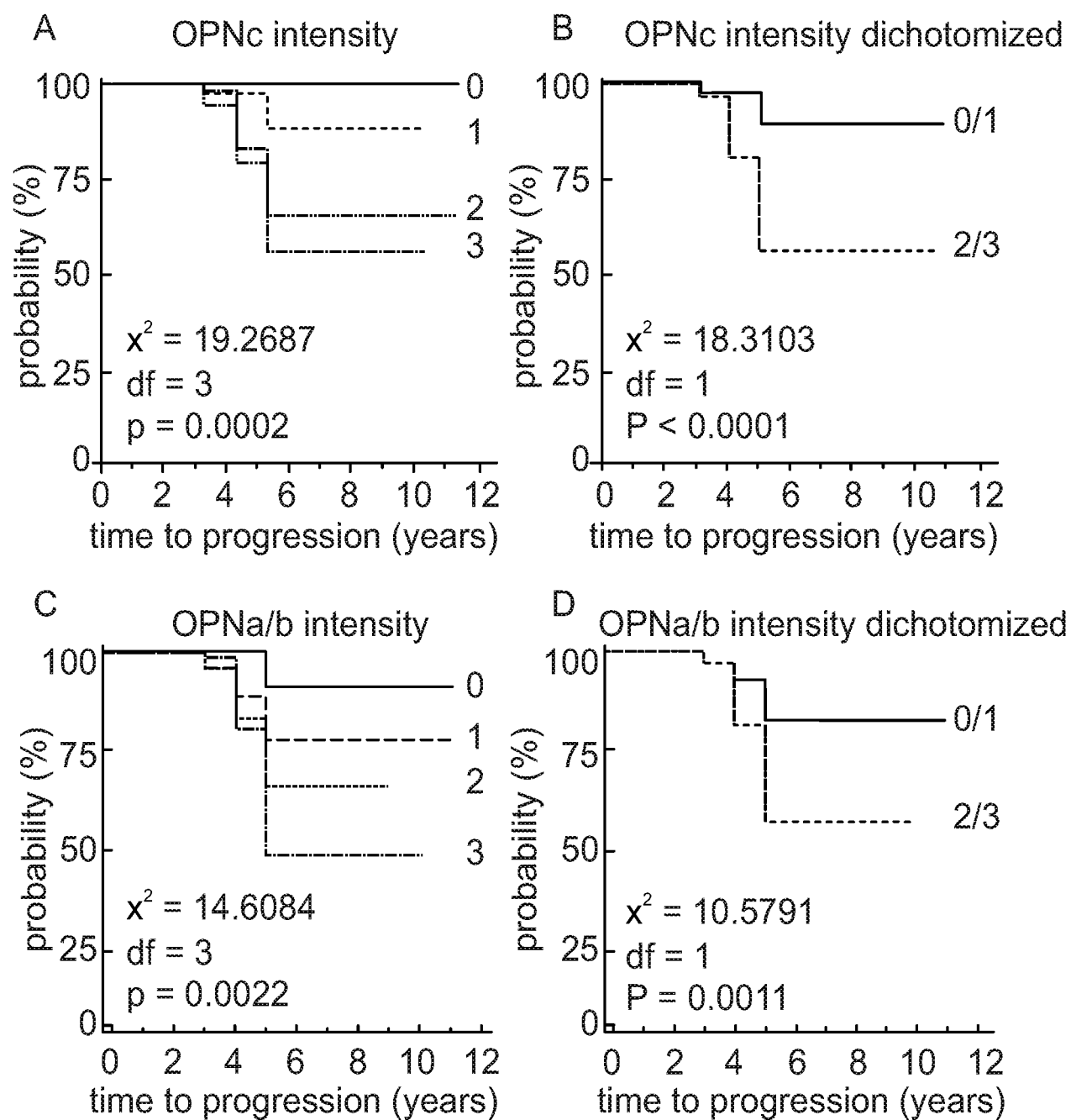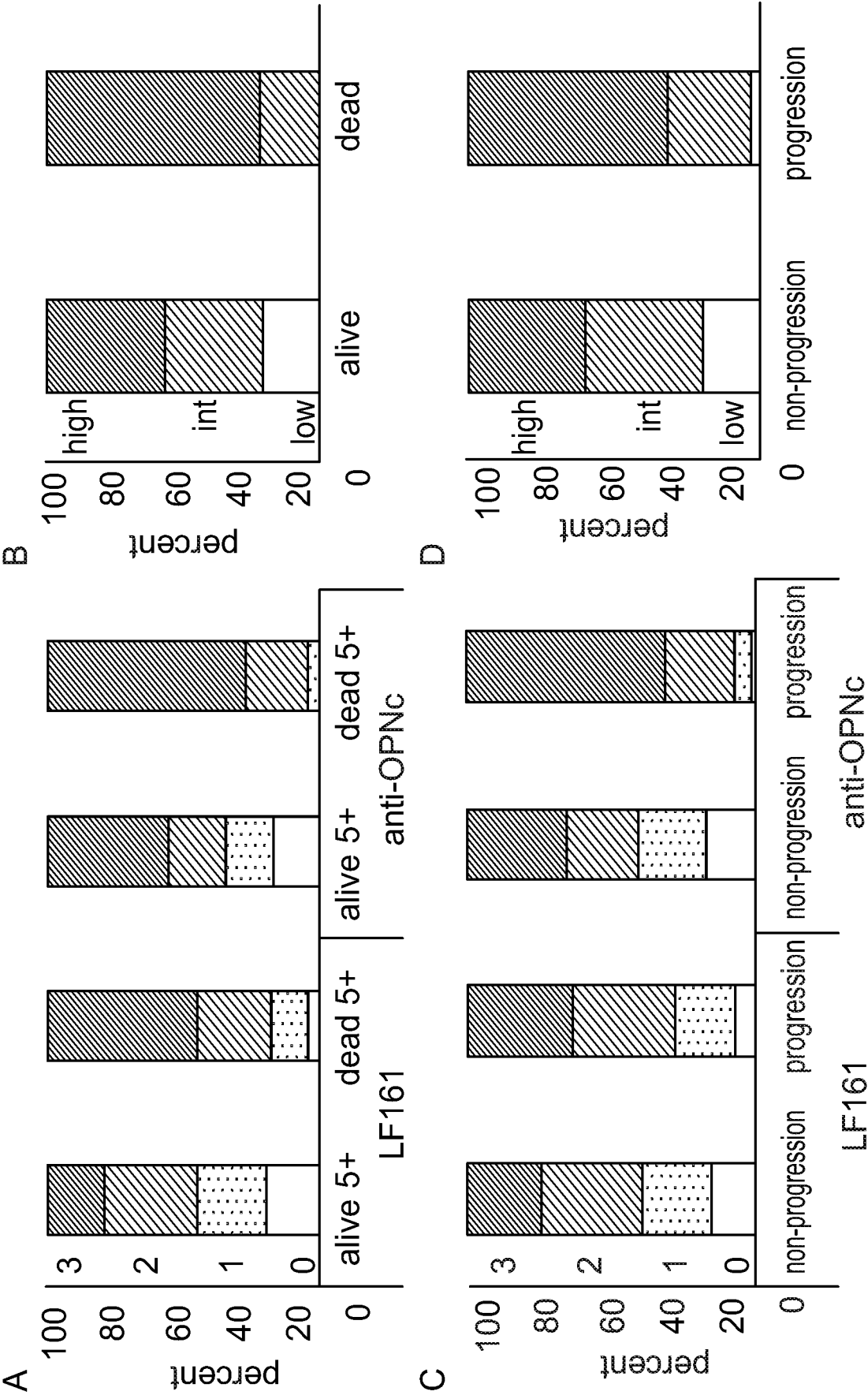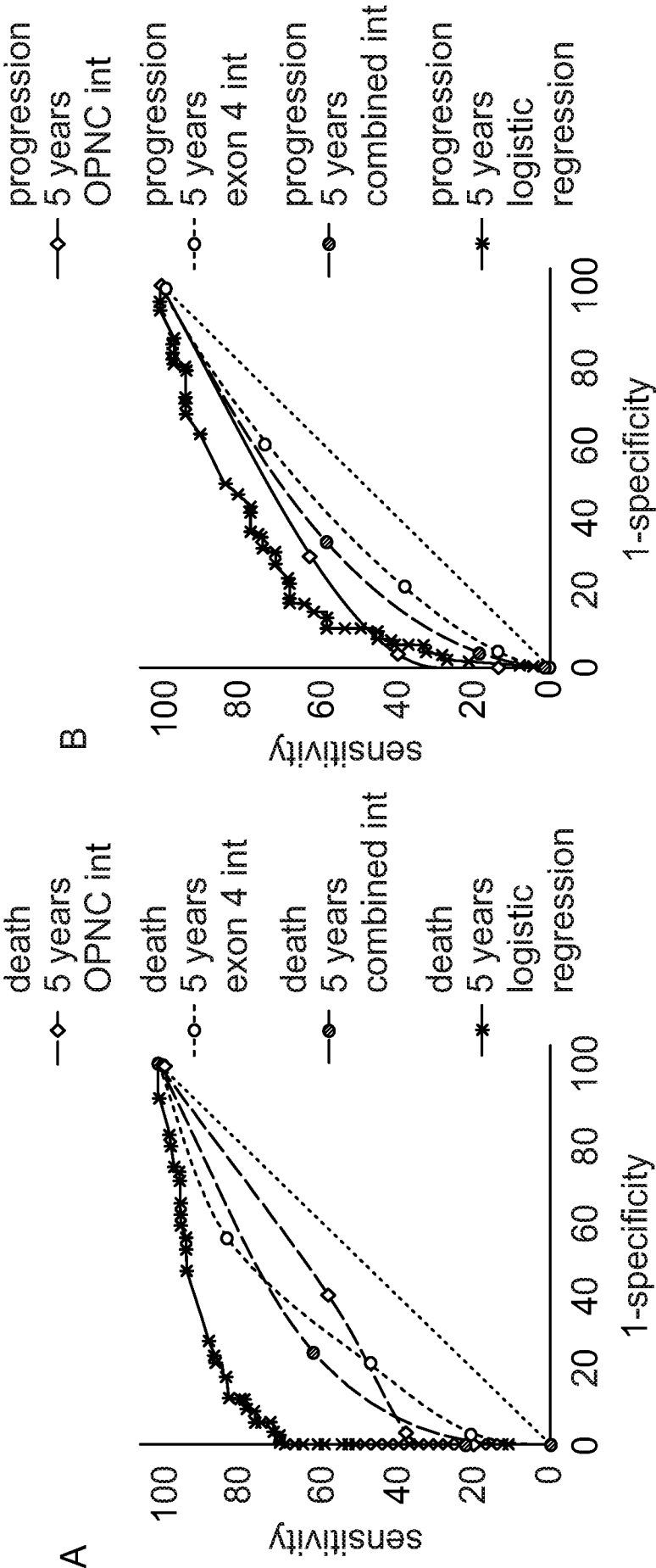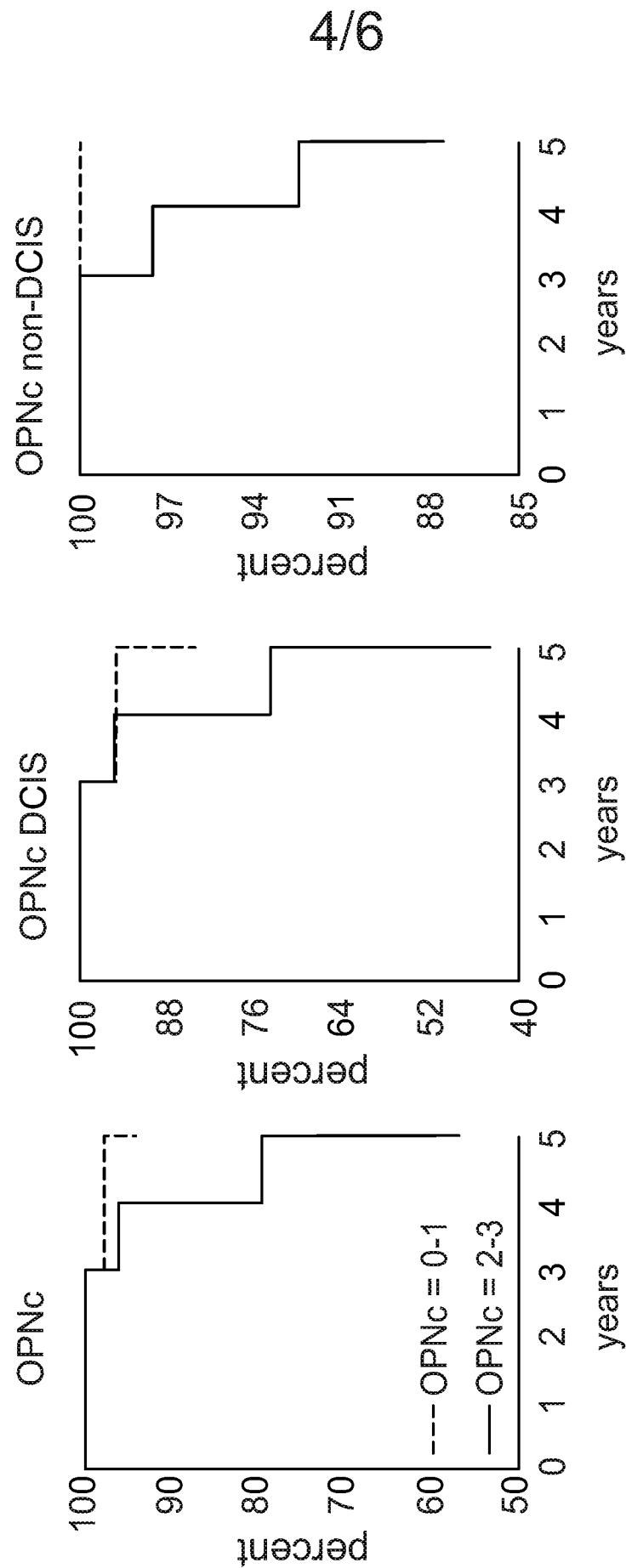
Fig. 1

Fig. 2

3/6

Fig. 3

Fig. 4
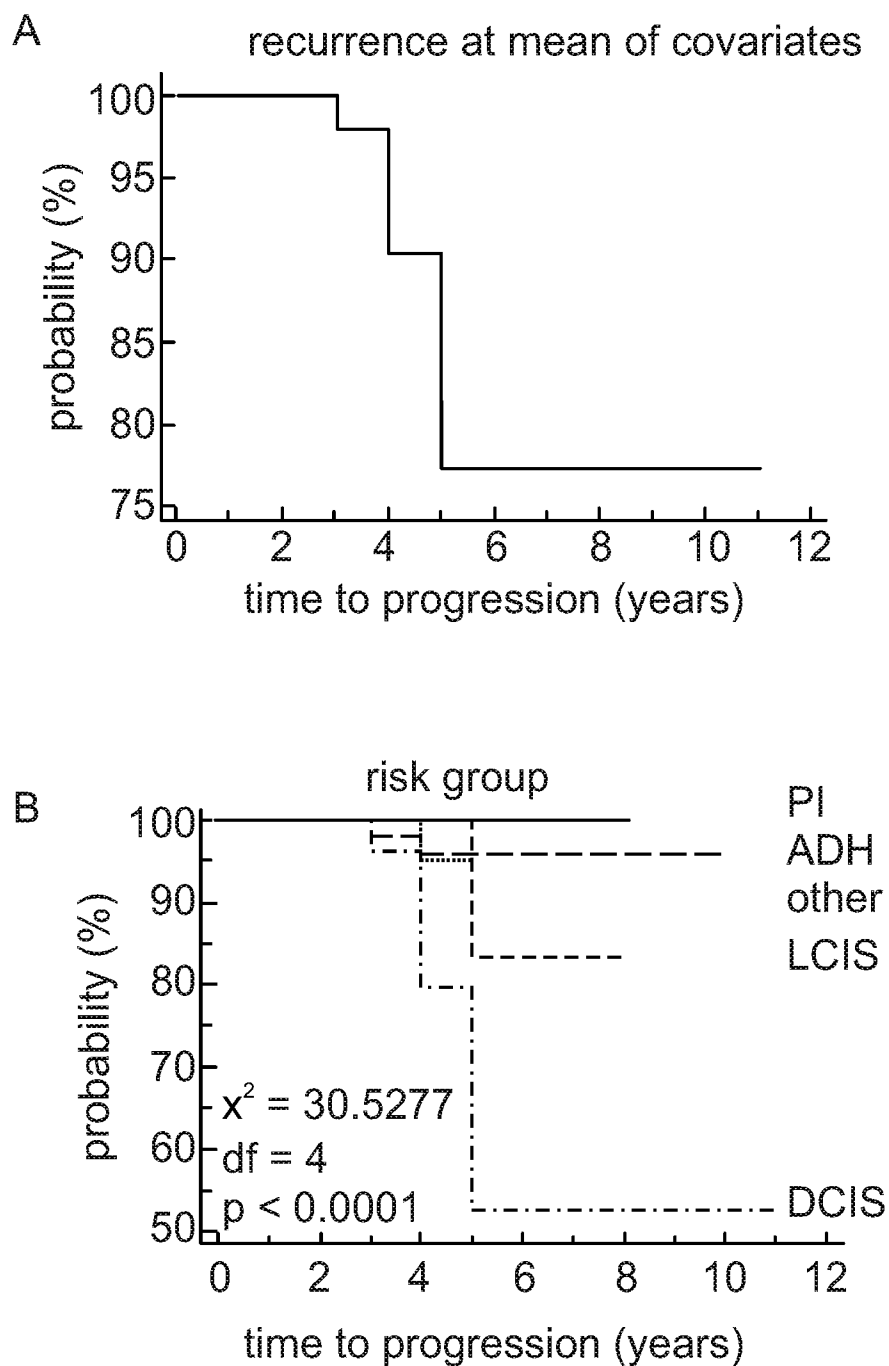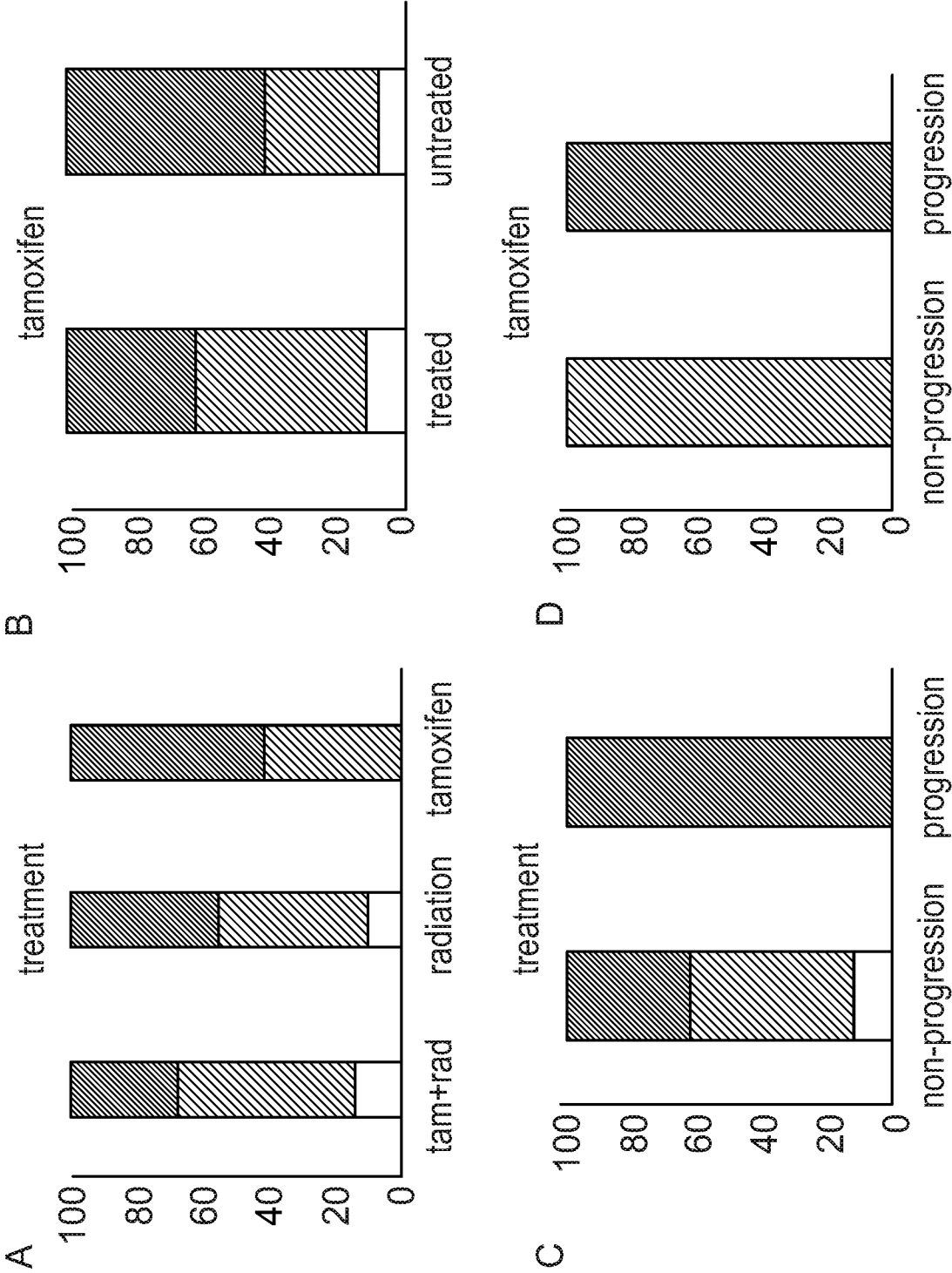
A

recurrence at mean of covariates

B

risk group

$x^2 = 30.5277$
df = 4
p < 0.0001

PI
ADH
other
LCIS

DCIS

Fig. 5

Fig. 6

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 14/52; C07K 16/18; C07K 16/24; C12Q 1/68; G01N 33/53; G01N 33/574 (2018.01)

CPC - A61K 2039/505; C07K 14/52; C12Q 1/68; C12Q 2600/106; C12Q 2600/112; G01N 33/574; G01N 2333/52; G01N 2800/50; G01N 2800/52 (2018.08)

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)

See Search History document

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

USPC - 424/139.1; 435/6; 435/375; 435/7.23; 435/7.1; 514/19.3; 530/387.1 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0209928 A1 (MIRZA et al) 19 August 2010 (19.08.2010) entire document	1-3, 6, 7, 17-19
Y		4, 5, 12-15
X	US 2009/0061436 A1 (WEBER) 05 March 2009 (05.03.2009) entire document	23-25
Y		5
Y	ZDUNIAK et al. "Nuclear osteopontin-c is a prognostic breast cancer marker," British Journal of Cancer, 27 January 2015 (27.01.2015), Vol. 112, Pgs. 729-738. entire document	4, 5, 12-15
A	BRIONES-ORTA et al. "Osteopontin splice variants and polymorphisms in cancer progression and prognosis," Biochim Biophys Acta Rev Cancer, 28 February 2017 (28.02.2017), Vol. 1868, Pgs. 93-108. entire document	1-25
A	US 2011/0165697 A1 (LIAW et al) 07 July 2011 (07.07.2011) entire document	1-25
A	US 2010/0291125 A1 (WINSLOW et al) 18 November 2010 (18.11.2010) entire document	1-25
A	ZDUNIAK et al. "Osteopontin splice variants are differential predictors of breast cancer treatment responses," BMC Cancer, 11 July 2016 (11.07.2016), Vol. 16:441, Pgs. 1-12. entire document	1-25

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"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
"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	"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
"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)	"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
"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	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 November 2018	**19 NOV 2018**

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450	Blaine R. Copenheaver
Facsimile No. 571-273-8300	PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

Box No. I   Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

   ☒ in the form of an Annex C/ST.25 text file.

   ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

   ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

   ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-8 were searched.

Form PCT/ISA/210 (continuation of first sheet (1)) (January 2015)

专利名称(译)	乳腺损伤的早期预测程序

公开(公告)号	EP3679061A1	公开(公告)日	2020-07-15
申请号	EP2018854610	申请日	2018-09-06

申请(专利权)人(译)	美国辛辛那提大学
当前申请(专利权)人(译)	美国辛辛那提大学
[标]发明人	WEBER GEORG F
发明人	WEBER, GEORG F.
IPC分类号	C07K14/52 C07K16/18 C07K16/24 C12Q1/68 G01N33/53 G01N33/574
CPC分类号	C07K14/52 C07K16/24 G01N33/53 G01N33/574 G01N33/57415 G01N33/6893 G01N2333/52 G01N2800/50
代理机构(译)	MOORE，MICHAEL RICHARD
优先权	62/554712 2017-09-06 US
外部链接	Espacenet

摘要(译)

提供了一种用于在诊断出患有病变的乳腺癌前病变并发展为乳腺癌的患者中预后风险的方法,该方法包括:提供该癌前病变的组织样品;以及 检测样品中选自OPN-a,OPN-b和OPN-c的骨桥蛋白(OPN)的一种或多种变体;如果检测到OPN-b和/或OPN-c和/或OPN-a升高到组织样本中的正常水平以上,则预示病变发展为乳腺癌的风险增加。 本文还提供了评估乳腺癌死亡风险的方法,治疗方法以及用于预后的试剂盒。