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(54) Title: METHODS OF SUBCLASSIFICATION OF DUCTAL CARCINOMA OF THE BREAST

(57) Abstract: Provided herein are methods of determining the aggressiveness or indolence of a ductal carcinoma in situ lesion. Also provided are methods of developing treatment plans for subjects with a ductal carcinoma in situ lesion based on the aggressiveness of the lesion.

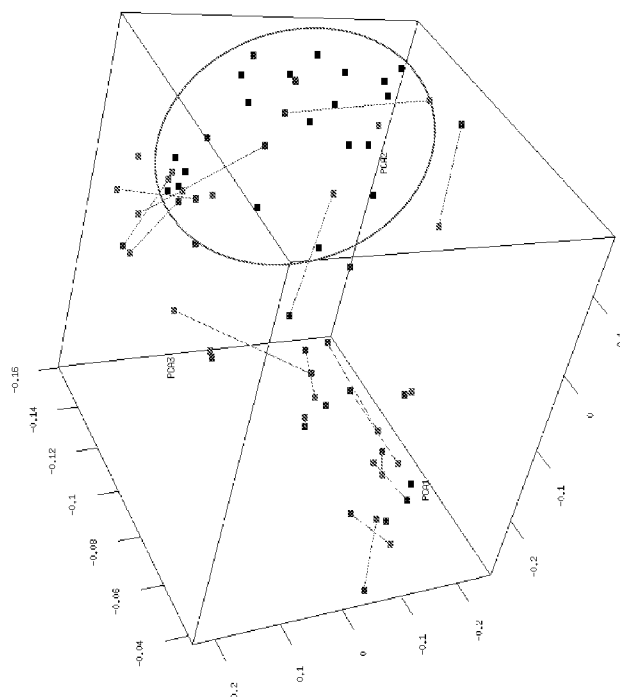


FIG. 1



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METHODS OF SUBCLASSIFICATION OF DUCTAL CARCINOMA OF THE BREAST**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims the benefit of U.S. Provisional Patent Application No. 61/158,583
5 filed March 9, 2009, which is incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant number R21CA 106676-01A1 awarded by the National Cancer Institute. The government has certain rights in the invention.

10

BACKGROUND

Ductal carcinomas in situ (DCIS) accounts for about 20% of newly diagnosed breast cancers in the United States amounting to nearly 40,000 cases per year. DCIS is characterized by marked pathologic and clinical heterogeneity. Only a minority of DCIS have the capacity to progress to stromally invasive tumors. From 30-50% of high grade (HG) DCIS lesions progress to
15 invasive carcinoma. In contrast, only about 10-20% of low grade (LG) DCIS progress to an invasive carcinoma. Standard pathologic evaluation does not distinguish DCIS lesions with or without the capacity to progress. In addition, no molecular changes have been identified that reliably differentiate indolent from aggressive DCIS.

As there are no distinguishing features of indolent DCIS, the great majority of patients tend
20 to be over-treated. Patients with a diagnosis of DCIS receive additional surgery, radiation or systemic therapy. Such aggressive treatments are not necessary in 80-90% of LG DCIS and in 50-70% of HG DCIS cases.

SUMMARY OF THE INVENTION

In one aspect, methods of determining the aggressive potential of a ductal carcinoma in situ (DCIS) lesion are provided. The DNA copy number, the level of mRNA expression of a gene or the level of protein expression from a gene in a sample from the DCIS lesion is detected and the results are used to determine the aggressiveness or indolence of the DCIS lesion. The gene may be any or a combination of the genes encoding NR4A1, NCOR2/SMRT, DYNLRB2, TAF1C, GRAP2, ST13, CELSR1, or UPK3A. The results may be used to develop a treatment plan for the subject with the DCIS lesion.

In another aspect, methods for classifying aggressiveness or indolence of a ductal carcinoma in situ (DCIS) lesion are provided. The methods include detecting a DNA copy number of at least one nucleic acid in a cell from the DCIS lesion and using the DNA copy number to classify the aggressiveness or indolence of the DCIS lesion. The nucleic acid is at a human chromosomal location selected from 22q13.1-q13.33, 16q23-q24.2, 12q13-q14 or 12q24-q25.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a three-dimensional graphical plot showing the principal component analysis (PCA) for 20 PD (black), 25 MD (blue) and 24 MI (red) lesions (where PD indicates pure (indolent) DCIS, MD mixed (aggressive) DCIS, and MI matched invasive carcinoma). 18 cases had both an MD and MI component and are connected by lines. The circle contains all but one of the PD lesions, indicating that they share genomic alterations that set them apart from the aggressive (MD) DCIS lesions.

Figure 2 is a set of graphs showing the DNA copy number changes in PD (A) and MD (D) lesions by their chromosomal location.

Figure 3 is a heat map of chromosomal are 22q. The x-axis shows the 20 PD lesions on the left and the 25 MD lesions on the right. The scale on the right indicates the $-\log_{10}$ P-values for differential amplification (red) or deletion (green) in the two groups. The y-axis shows the

chromosomal location from the telomere (top) to the centromere (bottom). Near the telomere gains are more common in PD. The remainder of the arm shows higher frequencies of deletions in MD lesions.

Figure 4 is a set of graphs showing the analysis of the DNA copy number experiments using 5 and 8 genes combinations and the ROC curves and AUCs for these experiments.

DETAILED DESCRIPTION

The Examples below demonstrate that the DNA copy number of particular genes found in particular regions of several chromosomes are likely to be altered in ductal carcinoma in situ (DCIS) lesions and that the copy number of the gene in cells from the lesion is indicative of the aggressiveness of the cancer. The Examples demonstrate ductal carcinoma invasiveness can be predicted by analysis of the DNA copy number at a human chromosomal location selected from 22q13.1-q13.33, 16q23-q24.2, 12q13-q14 or 12q24-q25. The DNA copy number was shown to be increased in indolent DCIS at 22q13.1-q13.33, 16q23, 12q13-q14 or 12q24-q25 relative to controls. The DNA copy number was more likely to be increased at 22q13.1 relative to controls in DCIS lesions that were aggressive and likely to become invasive. The DNA copy number was more likely to be reduced at 16q23, 12q13-q14 or 12q24-q25 in aggressive DCIS lesions relative to controls. The DNA copy number was more likely to be reduced at 16q24.1 in indolent DCIS relative to controls.

Control or normal cells are expected to have two alleles of each gene. Four or more copies of a gene is considered an increase relative to control cells and one or no copies of a gene is considered as a decrease in copy number relative to controls. The number of DNA copies of the gene can be detected in a sample from the DCIS lesion.

The sample may be obtained by any means available to those skilled in the art including, but not limited to, a biopsy of the DCIS lesion, including a needle biopsy or surgical removal of

tissue containing the lesion. Breast tissues with DCIS generally contain normal cells as well as neoplastic cells. In the Examples, the cells used in the samples for preparation of the DNA for aCGH were microdissected to ensure that only DCIS cells were included. As noted in the Examples, the gene copy number may be assessed in samples including both DCIS cells and
5 normal cells. The percentage of DCIS vs. normal cells can be used to calculate whether the DCIS cells have a change in gene copy number as compared to non-neoplastic cells.

Gene copy number may be assessed by any method, including methods known to those of skill in the art. For example the gene copy number may be assessed by methods, including but not limited to, array comparative genomic hybridization (aCGH), in situ hybridization (ISH, e.g.,
10 fluorescent (FISH) or chromogenic (CISH)), microarray analysis and real time quantitative PCR. Control cells for comparison may be normal, non-neoplastic cells from the same or a different subject or may be cancer cells with known genomic DNA changes and defined aggressiveness. As noted above, when assessing gene copy number, control cells are not needed for comparison due to the diploid nature of the human genome.

15 Several specific genes whose gene copy number was predictive of the DCIS lesion's aggressiveness (i.e. the propensity of a lesion to become invasive) were also identified in the Examples. In particular, increased copy number of NCOR2/SMRT (chromosome location 12q24.31), NR4A1 (chromosome location 12q13), DYNLRB2 (chromosome location 16q23.3), CELSR1 (chromosome location 22q13.31), UPK3A (chromosome location 22q13.31) or ST13
20 (chromosome location 22q13.1) was shown to be indicative of indolent or non-aggressive DCIS lesions. Conversely a decreased copy number of NCOR2/SMRT, NR4A1, DYNLRB2 was indicative of increased aggressiveness and a higher likelihood of invasiveness. A decrease in ST13 copy number was indicative of an indolent lesion. An increase in GRAP2 (chromosome location 22q13.1) copy number in a DCIS cell was indicative of increased aggressiveness. A reduced copy

number of TAF1C (chromosome location 16q24.1) in a DCIS lesion was indicative of an indolent lesion. The GenBank NCBI and Ensembl Gene ID No. for the genes are included in Table 1.

Table 1: GenBank and Ensembl Gene ID No.

Gene	NR4A1	NCOR2	DYNLRB2	TAF1C	GRAP2	ST13	CELSR1	UPK3A
NCBI: GeneID:	3164	9612	83657	9013	9402	6767	9620	7380
Ensembl Gene ID	ENSG00000123358	ENSG00000196498	ENSG00000168589	ENSG000001013168	ENSG00000100351	ENSG000001100380	ENSG00000105275	ENSG000001100373

5 As demonstrated in the Examples, overexpression of genes as evidenced by increased mRNA or protein levels due to increased gene copy number or conversely, reduced expression of genes as evidenced by decreases in mRNA or protein levels due to reduced gene copy number may be indicative of the aggressiveness or invasive potential of the DCIS lesion. Therefore, the mRNA or protein expression level of the genes as compared to control cells may also be used to assess the

10 aggressiveness of the DCIS lesion. Gene expression levels may be assessed either at the level of mRNA expression, e.g. by Northern blot or quantitative rtPCR, or at the level of protein expression, e.g. by Western blot, ELISA, immunohistochemistry (IHC) or functional protein assays. Other methods of assessing gene expression or protein expression may also be used, including additional methods known to those skilled in the art.

15 The DCIS lesion may be classified or predicted to be aggressive or indolent based on analysis of the DNA copy number, mRNA expression or protein expression of a single gene, the combination of all eight genes, or a sub-combination of a portion of the eight genes identified herein. As shown in the examples below, detection of a change in DNA copy number, mRNA levels or protein levels of one of the genes described herein may be capable of predicting the

20 likelihood of invasive carcinoma. Evaluation of NCOR2/SMRT alone could be used to determine the aggressiveness of the lesion. Evaluation and detection based on all eight genes identified herein was capable of determining the aggressiveness of the lesion with an AUC of 0.946. In addition, at least one set of five genes including NR4A, NCOR2/SMRT, DYNLRB2, GRAP2, and CELSR1

was capable of predicting the aggressiveness of the lesion with an AUC of 0.953. Any combination of the eight genes may be used in the methods described herein.

The determination of the aggressiveness phenotype of the DCIS lesion may be used to develop a treatment plan for the subject with the DCIS lesion. As noted above, most DCIS is indolent, but due to the propensity of some DCIS to become invasive many subjects with DCIS are treated aggressively. A reliable test to determine the propensity of a DCIS lesion to progress to invasive cancer would save many patients from undergoing needless procedures and would reduce health care costs. Those subjects whose lesions are likely indolent may be treated by monitoring the lesion over time. Those subjects whose lesions are likely aggressive can receive aggressive therapy, such as surgery, radiation, chemotherapy or a combination thereof. Thus, the results of the methods provided herein may be used to develop treatment plans for the subject or may be used as a prognostic assay to provide physicians with additional information.

EXAMPLES

Low grade (LG) DCIS cases were selected for analysis. For these studies low-grade DCIS was defined as nuclear grade 1 or 2 with no necrosis apparent. Of the 116 cases of formalin fixed, paraffin embedded (FFPE) LGDCIS samples selected, 61 of them contained a small invasive component, indicating aggressive potential (MD = mixed DCIS, MI = mixed invasive carcinoma) and 55 cases showed no evidence of invasion within at least 5 years (PD = pure DCIS). These samples were from the surgical pathology files of Duke University Medical Center (DUMC) and Roswell Park Cancer Institute (RPCI). The three different types of lesions were microdissected using laser capture microdissection (over 200 paraffin blocks; 10 μ m sections), genomic DNA was extracted, and 118 array comparative genomic hybridization (aCGH) hybridizations were performed utilizing high density (19k) bacterial artificial chromosome (BAC) arrays (average genomic resolution was 170kb). The arrays were custom made by the RPCI Genomics Core

Facility. The DNA quality was assessed via the Enzo BioScore Assay and samples with poor DNA quality were not used for further analysis.

Sixty-nine of the 118 aCGH hybridizations yielded interpretable DNA profiles: 20 PD, 25 MD, 24 MI ("Cohort 1"). Statistical analyses were performed using the generic GCH Call program. Surprisingly, while paired in situ and invasive carcinomas had comparable frequencies of DNA copy number gains and losses, pure intraductal carcinomas were characterized by a larger number of copy number changes, especially gains (Table 2). Moreover, Principal component analysis (PCA) showed that the "indolent" DCIS lesions clustered separately from the mixed carcinomas (circled area in [Figure 1](#)). [Figure 2](#) demonstrates that 17 regions on 13 chromosomal arms contained differential copy number aberrations (CNAs), almost all of which occurred more commonly in pure (indolent) DCIS lesions. The majority of these differential CNAs were DNA gains.

Table 2: DNA copy number frequencies in low grade DCIS with and without associated invasive ductal carcinoma (IDC)

	N	Average copy number gains	Average copy number losses	Average total copy number changes
DCIS without IDC [PD]	20	8.4%	8.2%	16.6%
DCIS with IDC [MD]	25	6.3%	5.5%	11.8%
Matched IDC [MI]	24	6.9%	6.1%	13.0%
TOTAL	69	7.0%	6.5%	13.5%

[Figure 3](#) details the differential copy number changes on chromosome 22q. Only this chromosomal arm contained a higher frequency of copy number losses in the MD group, suggesting possible inactivation of invasion suppressor genes on that chromosomal arm. From the 17 differentially amplified or deleted regions, 19 candidate genes were selected for further validation studies, based on statistical probability and functional interest. Of those, 8 were confirmed to be differentially amplified or deleted in PD versus MD lesions, utilizing quantitative (Q-)PCR on aliquots of genomic DNA. The primers used for the PCR are provided in Table 3. These 8 genes and the data obtained are summarized in Table 4. Of note, three of these genes have

not been implicated in human neoplasia before, and only SMRT was previously described in human breast cancer.

5 **Table 3: Primers used for PCR**

Gene	OLIGO	Sequence	Amplicon	Tm
NR4A1	Forward	CTGAGACCACCAGGAAGAGC (SEQ ID NO: 1)		59.99
	Reverse	TCCTCCTCACGATCTGCTTT (SEQ ID NO: 2)	99	59.95
NCOR2	Forward	TCCCAGCGTGACTCTTTTCT (SEQ ID NO: 3)		59.99
	Reverse	ACCGGTGCTATGAACCAGTC (SEQ ID NO: 4)	90	60
DYNLRB2	Forward	GGGTAGCGTTGTTGACATCC (SEQ ID NO: 5)		60.38
	Reverse	ACGGAGACCCCAGATTTACC (SEQ ID NO: 6)	93	60.19
TAFIC	Forward	GCAGGTCCCTCAGTTCTCAC (SEQ ID NO: 7)		59.84
	Reverse	ACTCCACCCTCACCAAACAC (SEQ ID NO: 8)	84	59.86
Grap2	Forward	CCTGTGTGGCATTACCCTCT (SEQ ID NO: 9)		59.99
	Reverse	CCCCTTTAAGAGCTGTCACG (SEQ ID NO: 10)	80	59.87
ST13	Forward	CCCCGACAGCTGTAATTCAT (SEQ ID NO: 11)		59.96
	Reverse	GTCGAGCACAACCTTCAGCA (SEQ ID NO: 12)	88	60.18
CELSR1	Forward	GGTGTCTAGCAGTGGGTGT (SEQ ID NO: 13)		60.03
	Reverse	AAAGCTGGAGGCAAAAGACA (SEQ ID NO: 14)	90	59.99
UPK3A	Forward	GCCCACATTTTCATTTTGCT (SEQ ID NO: 15)		59.94
	Reverse	TGTAAGACGAAGGGGTTTGG (SEQ ID NO: 16)	100	59.96

Table 4: Differential copy number changes in low grade DCIS with (MD) and without (PD) associated stromal invasion: Eight candidate genes validated by Q-PCR

Candidate Gene	Chr. Loc.	Differential CNA	Function	Involvement in Human Neoplasia
SMRT (NCOR2)	12q24.31	More commonly amplified in PD, more commonly deleted in MD	Component of repressing nuclear receptor complex; involved in DNA repair	Expression correlated with breast cancer recurrence
NR4A1 (Nur77)	12q13.13	More commonly amplified in PD, more commonly deleted in MD	Nuclear steroid hormone receptor; pro-apoptotic	Downregulated in AML and in follicular thyroid ca
DYNLRB2 (DNLC2B)	16q23.2	More commonly amplified in PD, more commonly deleted in MD	Dynein light chain protein	Downregulated in liver cancer
TAF1C	16q24.1	More commonly deleted in PD	Transcription co-factor	Not described
CELSR1	22q13.31	More commonly amplified in PD	Flamingo-type cadherin; component of Wnt signaling pathway	Not described
UPK3A	22q13.31	More commonly amplified in PD	Cell membrane protein; involved in urothelial differentiation	Downregulation associated with worse prognosis in bladder cancer
ST13 (HIP)	22q13.1	More commonly amplified or deleted in PD	Hsp70 co-chaperone	Downregulated in colon cancer
GRAP2	22q13.1	More commonly amplified in MD	Involved in TCR signaling	Not described

Statistical analysis of the Q-PCR data showing the ROC curves and the AUC for combinations of 5 or 8 of the identified genes demonstrates that a change in gene copy number of at least 5 of these genes can discriminate between indolent and aggressive DCIS with high accuracy (Figure 4).

Because SMRT was the gene with the single highest discriminatory power, its differential copy number distribution was confirmed in an independent series of PD and MD lesions ("Cohort 2") using quantitative PCR. Cohort 2 consisted of 10ng samples from microdissected cells from a separate set of 18 PD and 17 MD lesions. The PCR assay sensitivity cut-off was +1.0 for gains and -0.5 for losses (log2 ratios). GAPDH was used as a reference gene. Table 5 details the results of three experiments. Each experiment demonstrated that SMRT gains were more common in PD lesions. In addition, the Q-PCR experiments revealed a much higher frequency of allele loss in the MD group. These findings suggest that SMRT may act as an invasion suppressor: it may be activated in DCIS lesions that do not have the capacity to invade and inactivated in lesions that do progress.

Table 5: SMRT copy number changes in PD vs. MD

	PD			MD			P-Value*
	Loss	Normal	Gain	Loss	Normal	Gain	
aCGH Cohort 1	0	5	15	2	17	6	0.0028
Q-PCR Cohort 1	6	10	4	22	3	0	0.0003
Q-PCR Cohort 2	4	10	4	11	6	0	0.0182

*Unordered Kruskal-Willis test

Gene copy number changes may correlate with a change in gene expression as measured by mRNA and/or protein expression levels in the cell. To test whether the protein expression levels correlated with the gene copy number immunohistochemistry (IHC) assays were performed on the tissue sections for NCOR2/SMRT protein levels. The paraffin sections were reacted with

mouse monoclonal antibody 1542/H7 from Santa Cruz at 2 µg/ml for 1 hour. The detection reaction utilized the Bond Refine horseradish peroxidase system from Leica.

The results are shown in Table 6. Although the changes are not statistically significant due to the small number of interpretable samples (n = 30), the results correlate to those obtained by aCGH and Q-PCR. The data suggest that NCOR2/SMRT levels are increased in a higher proportion of PD lesions (30% vs. 10%) and reduced or absent in a higher proportion of MD lesions (20% vs. 10%).

Table 6: IHC of PD and MD lesions for NCOR2/SMRT protein levels

DCIS Type	No/Reduced Expression	Normal Expression	Increased Expression
PD	1	6	3
MD	4	14	2

Prophetic Examples

In the Examples above, pure populations of DCIS cells were isolated via laser capture microdissection of paraffin sections, and genomic DNA was prepared and assayed from those preparations following routine protocols. For each of the 8 genes comprising the “invasion signature”, appropriate primers and Q-PCR protocols were designed. GAPDH was used as a reference gene with normal copy number and defined thresholds of gains of at least two alleles, and of losses of at least one allele. However, tissue microdissection is laborious and requires specialized equipment. Whole tissue sections from the same cohort of cases from which the signature was derived will be used to determine if microdissection is necessary. Briefly, from whole tissue sections, genomic DNA will be extracted in the same fashion, and Q-PCR experiments will be performed using the established protocols. Because the tumor DNA will be diluted by DNA from non-neoplastic cells, the amplitude of copy number gains and losses is

expected to be lower for each case. To account for this effect and to set appropriate thresholds that define copy number gains and losses, we will perform dilution curves for each gene. One sample with a 2x copy number, and another one with a defined loss of one allele, will be diluted with DNA extracted from formalin fixed, paraffin embedded normal human breast tissue to yield ratios of 25%, 50%, and 75%, reflecting the typical range of tumor cellularity in whole tissue sections. The evaluation of copy number over the various dilutions will be observational, and descriptive statistics and Spearman rank correlation will be used to determine threshold values for copy number losses and gains.

The differential copy number gains and losses were defined using LGDCIS. We hypothesize that similar molecular changes exist in HGDCIS lesions (i.e., intraductal carcinomas with a nuclear grade of 3) as well. To this end, we will utilize 50 HGDCIS cases from the DUMC files and/or from other sources. The experiments and statistical analysis will be performed as described above.

A chromogenic in situ hybridization (CISH) assay for all 8 genes will be developed. This technique also detects DNA copy number changes, but allows evaluation specifically of the neoplastic cell population. The procedure for CISH is very similar compared to the more commonly used FISH technique, except that the detection reaction utilizes chromogenic substrates instead of fluorescent dyes. CISH has several advantages, including use of brightfield microscopy and permanence of the signals. These assays may be routinely performed in a diagnostic laboratory. For each of the 8 genes, we will construct appropriate biotinylated oligonucleotide probes (Integrated DNA Technologies, Skokie, IL). We will employ the Dako GenPoint kit to detect the presence or absence of specific hybridization (Dako, Carpinteria, CA). Briefly, the slides will be deparaffinized and heated in target retrieval buffer (sub-boiling for 20 min). They will be quenched in 3% H₂O₂ in methanol for 20 min. The DNA will be denatured at

95°C for 5 min. For each biotinylated oligonucleotide probe, the optimal hybridization and stringent wash temperature will be determined. After incubation in the hybridizer instrument, the washed slides will be reacted with primary streptavidin-HRP solution for 15 min. A subsequent amplification step utilizes biotinyl tyramide (15 min). This will be followed by a second streptavidin-HRP step (15 min). The chromogenic reaction utilizes DAB (5 min). Finally, the slides will be counterstained with hematoxylin and mounted for light microscopic evaluation. For any given gene, the number of signals in each tumor cell will be counted. We will evaluate 100 DCIS cells per case. An average signal number of 4 or more per cell will count as a gain; an average signal number of 1 or less will count as a loss. As controls, we will use benign tissues from non-cancer patients. Normal cells should contain 2 copies of each gene. Negative control reactions will be performed without DNA probes. When all genes have been scored in all samples, statistical analysis will be performed to determine which combination of the 8 genes has the highest power of differentiating DCIS cases with and without the capacity to invade the stroma, using the same additive and multivariate models described above.

As an alternate in situ technique we will employ IHC, which interrogates gene expression at the protein level. It is reasonable to expect that DNA copy number gains will be associated with increased, and losses with decreased, levels of the corresponding protein product. For 7 of the 8 genes in our panel, commercial antibodies are available (at present, no antibodies exist against DYNLRB2). We previously used the SMRT antibody to develop an IHC assay to study expression of that gene in invasive breast carcinomas; we found downregulation or loss in 80% of cases. Indeed, in the Examples above the SMRT protein levels were found to correlate to the gene copy number. Briefly, 5µm paraffin sections are dewaxed and rehydrated. For each antibody, the optimal antigen retrieval method (e.g. enzymatic, heat induced, combination) is established. The optimal antibody titer and incubation time and temperature are determined, using known positive and negative controls. These include numerous well characterized human

cancer cell lines that have been fixed and paraffin embedded, as well as a large number of archival normal and neoplastic tissues. Several different detection methods are available. We routinely use peroxidase based detection systems including the Vectastain Elite ABC kit (Vector), the mouse and rabbit Envision kits (Dako), and the LSAB kit (Dako). For antigens
5 expressed at low levels, we can employ a signal amplification step (Dako). DAB is used as chromogen and hematoxylin as counterstain. Nonspecific species-matched antibodies are used in negative control reactions. Evaluation of the IHC stains will take into account the subcellular localization, percentage of staining cells, staining pattern (diffuse, mosaic, patchy, focal) and staining intensity.

10

CLAIMS

1. A method of determining the aggressiveness of a ductal carcinoma in situ (DCIS) lesion comprising:

(a) detecting the DNA copy number, the level of mRNA expression of a gene or the level of protein expression from a gene in a sample from the DCIS lesion, wherein the gene is selected from the genes encoding NR4A1, NCOR2/SMRT, DYNLRB2, TAF1C, GRAP2, ST13, CELSR1, or UPK3A; and

(b) using the results of step (a) to determine the aggressiveness of the DCIS lesion.

2. The method of claim 1, wherein an increased DNA copy number, mRNA expression or protein expression of NR4A1, NCOR2/SMRT, DYNLRB2, CELSR1 or UPK3A is indicative of an indolent DCIS lesion.

3. The method of claim 1, wherein a decreased DNA copy number, mRNA expression or protein expression of NR4A1, NCOR2/SMRT, or DYNLRB2 is indicative of an aggressive DCIS lesion.

4. The method of claim 1, wherein an increased DNA copy number, mRNA expression or protein expression of GRAP2 is indicative of an aggressive DCIS lesion.

5. The method of claim 1, wherein a decreased DNA copy number, mRNA expression or protein expression of TAF1C is indicative of an indolent DCIS lesion.

6. The method of claim 1, wherein a change in the DNA copy number, mRNA expression or protein expression of ST13 is indicative of an indolent DCIS lesion.

7. The method of any of claims 1-6, wherein the DNA copy number, mRNA expression or protein expression of NR4A1, NCOR2/SMRT, DYNLRB2, TAF1C, GRAP2, ST13, CELSR1 and UPK3A are detected.

8. The method of any of claims 1-6, wherein the DNA copy number, mRNA expression or protein expression of NR4A1, NCOR2/SMRT, DYNLRB2, TAF1C, GRAP2, ST13, and CELSR1 are detected.
9. The method of any of claims 1-6, wherein the DNA copy number, mRNA expression or protein expression of NR4A1, NCOR2/SMRT, DYNLRB2, GRAP2, and CELSR1 are detected.
10. The method of any of claims 1-6, wherein the DNA copy number, mRNA expression or protein expression of NR4A1, TAF1C, GRAP2, and ST13 are detected.
11. The method of any of claims 1-6, wherein the DNA copy number, mRNA expression or protein expression of NCOR2/SMRT, DYNLRB2, UPK3A and CELSR1 are detected.
12. The method of any of claims 1-11, wherein the sample is a DCIS cell.
13. The method of claim 12, wherein the sample includes cells not from the DCIS lesion.
14. The method of any of claims 1-13, wherein the DNA copy number is detected by array comparative genomic hybridization (aGCH), quantitative PCR, or in situ hybridization.
15. The method of any of claims 1-13, wherein the mRNA expression is detected by quantitative rt-PCR, microarray analysis, Northern blot, or dot blot.
16. The method of any of claims 1-13, wherein the protein expression is detected by Western blot, immunohistochemistry, or ELISA.
17. The method of any of claims 1-16, wherein the aggressiveness of the DCIS lesion is used to develop a treatment plan for a subject with the DCIS lesion.
18. The method of claim 17, wherein the treatment plan comprises monitoring a subject with an indolent DCIS lesion.
19. The method of claim 17, wherein the treatment plan comprises surgery, radiation, endocrine therapy, chemotherapy, or a combination thereof for a subject with an aggressive DCIS lesion.
20. A method for classifying aggressiveness or indolence of a ductal carcinoma in situ (DCIS) lesion comprising detecting a DNA copy number of at least one nucleic acid in a cell from the

DCIS lesion, wherein the nucleic acid is at a human chromosomal location selected from 22q13.1-q13.33, 16q23-q24.2, 12q13-q14 or 12q24-q25 and using the DNA copy number to classify the aggressiveness or indolence of the DCIS lesion.

21. The method of claim 20, wherein the nucleic acid is selected from the polynucleotides
5 encoding NR4A1, NCOR2/SMRT, DYNLRB2, TAF1C, GRAP2, ST13, CELSR1, or UPK3A.

22. The method of claim 21, wherein an increased copy number of NR4A1, NCOR2/SMRT, DYNLRB2, CELSR1 or UPK3A is indicative of an indolent DCIS lesion.

23. The method of claim 21, wherein a decreased copy number of NR4A1, NCOR2/SMRT, or DYNLRB2 is indicative of an aggressive DCIS lesion.

10 24. The method of claim 21, wherein an increased copy number of GRAP2 is indicative of an aggressive DCIS lesion.

25. The method of claim 21, wherein a decreased copy number of TAF1C is indicative of an indolent DCIS lesion.

15 26. The method of claim 21, wherein a change in the copy number of ST13 is indicative of an indolent DCIS lesion.

27. The method of any of claims 21-26, wherein the DNA copy number of NR4A1, NCOR2/SMRT, DYNLRB2, TAF1C, GRAP2, ST13, and CELSR1 are detected.

28. The method of any of claims 21-26, wherein the DNA copy number of NR4A1, NCOR2/SMRT, DYNLRB2, GRAP2, and CELSR1 are detected.

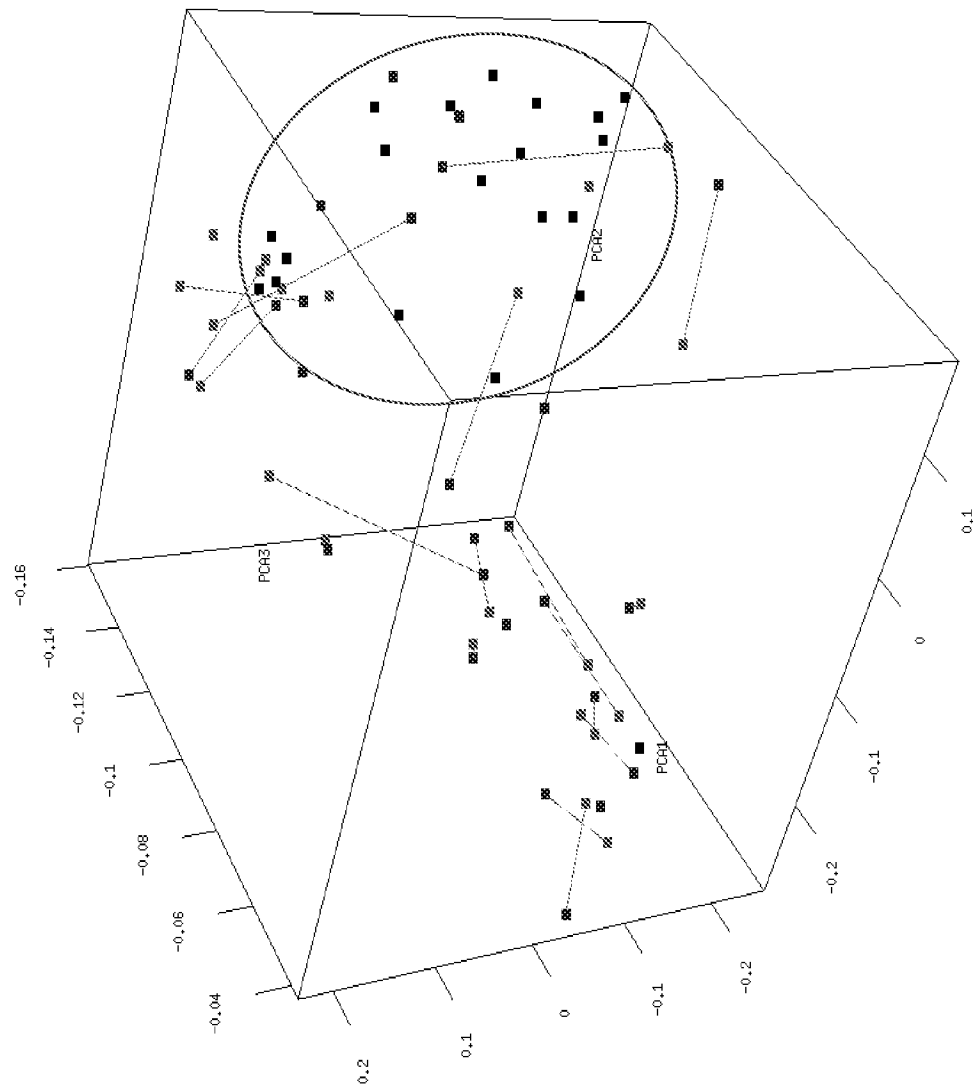
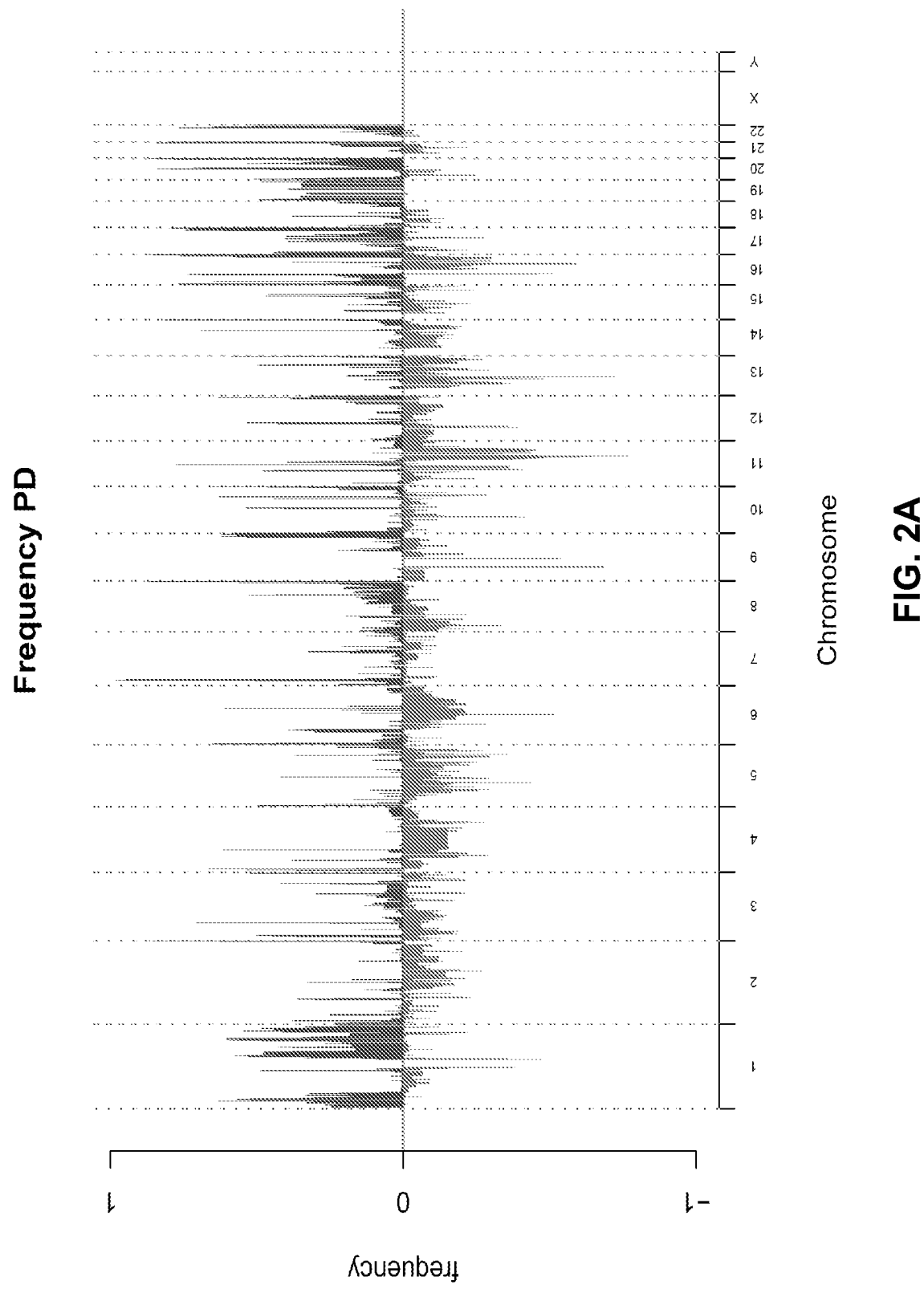


FIG. 1



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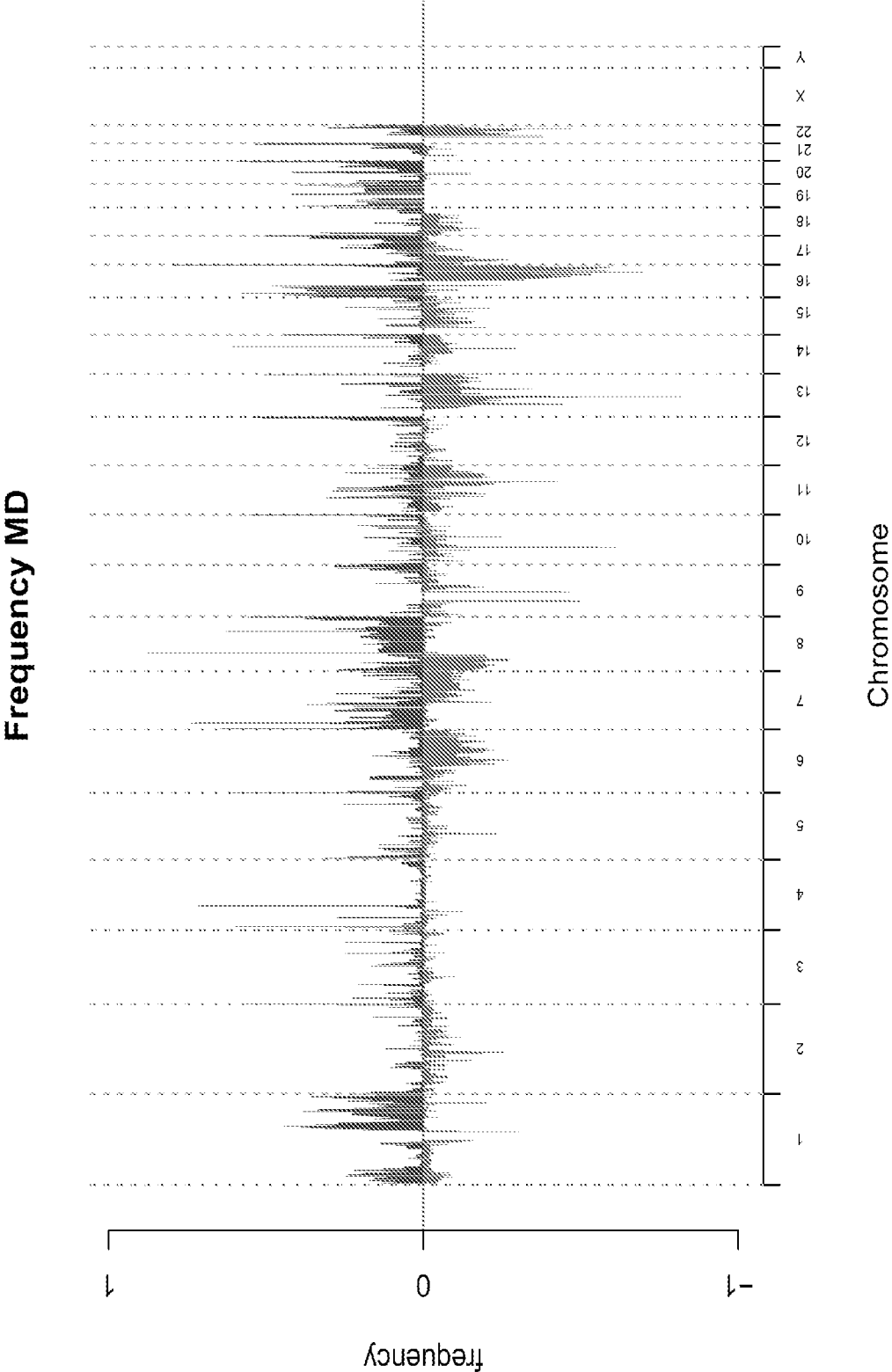


FIG. 2B

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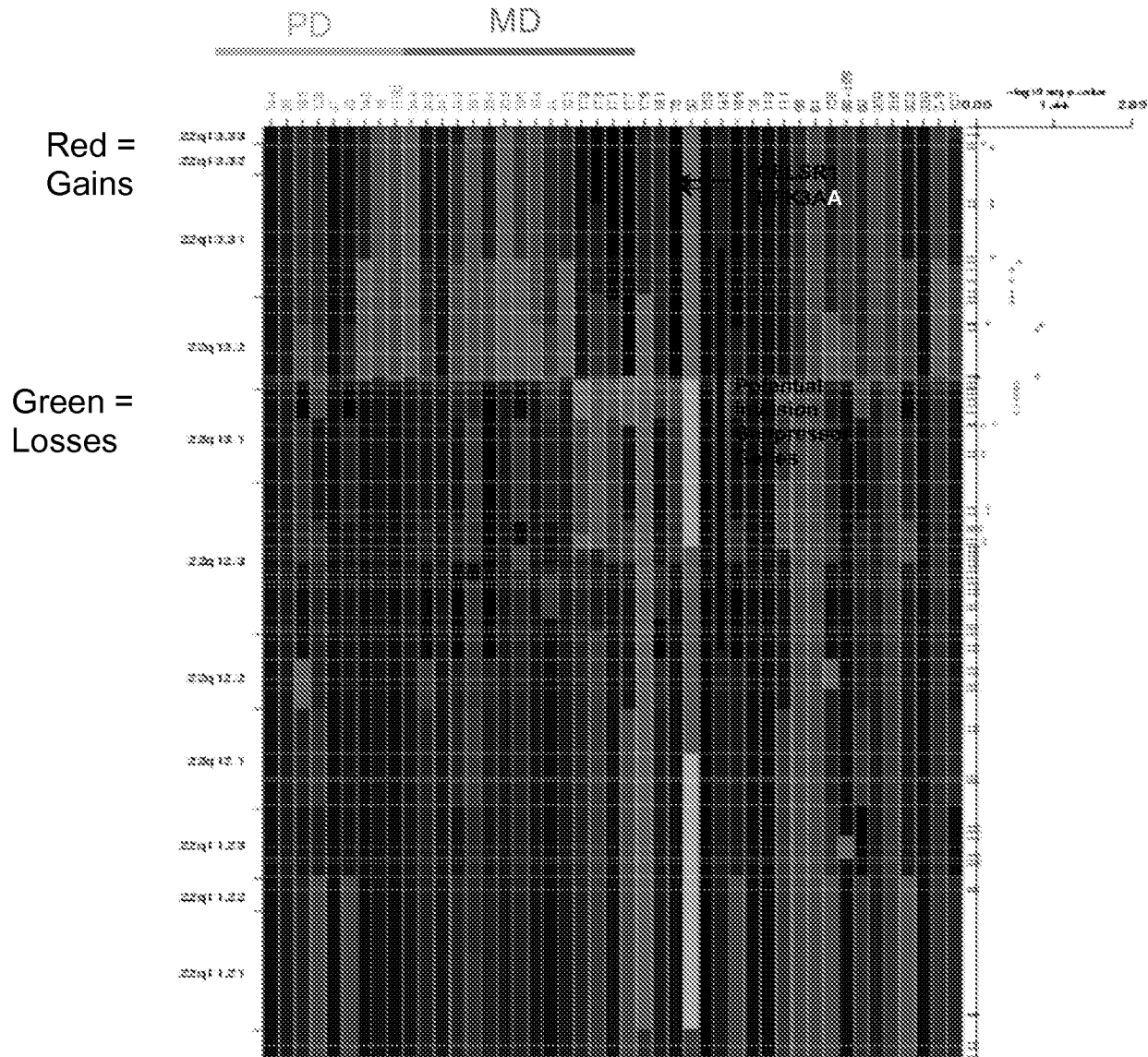


FIG. 3

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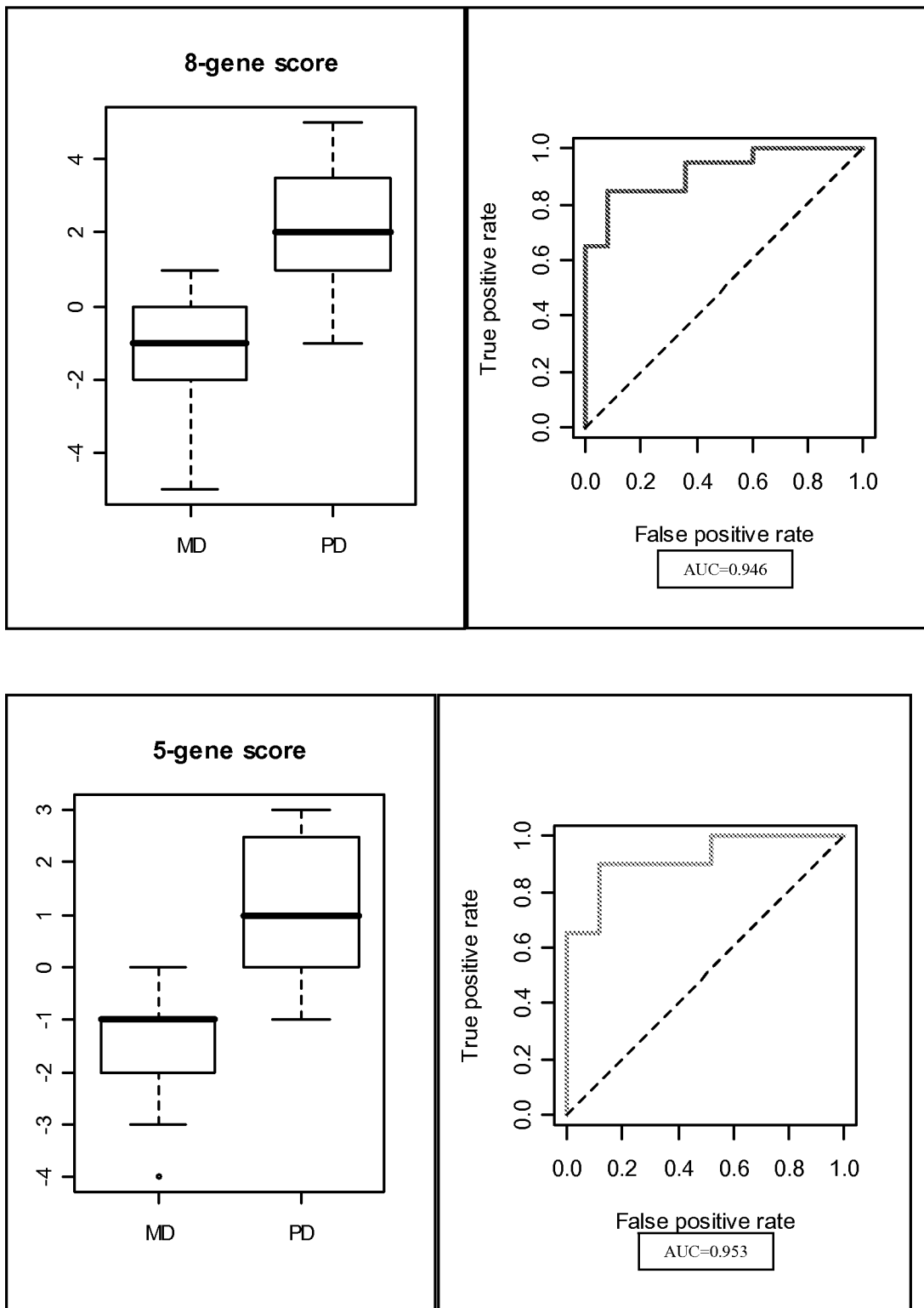


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/67722

A. CLASSIFICATION OF SUBJECT MATTER

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

USPC - 435/6; 436/64, 94

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: 435/6; 436/64, 94Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/6; 436/64, 94 (text search)Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PubWEST (PGPB, UPST, JPAB, EPAB); Google Scholar
Search terms: breast cancer, ductal carcinoma in situ (DCIS), invasive ductal carcinoma, expression profiling, microarray, NR4A1, NCOR2/SMRT, DYNLRB2, CELSR1, ST13, GRAP2, TAF1C, UPK3A

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	KUREBAYASHI et al. Expression Levels of Estrogen Receptor-alpha, Estrogen Receptor-beta, Coactivators, and Corepressors in Breast Cancer. Clin Can Res February 2000 Vol 6 No 2 Pages 512-518. Pg 516 fig 3, pg 517 left col para 1.	1-3 ----- 5, 6, 20-23, 25, 26 ----- 4, 7-11, 24, 27, 28
Y --- A	US 2008/0311573 A1 (LILLIE et al.) 18 December 2008 (18.12.2008) Especially para [0337], sheet 48 table 6.	5, 25 ----- 7-11, 27, 28
Y --- A	US 2004/0191783 A1 (LECLERCQ et al.) 30 September 2004 (30.09.2004) Especially para [0024]	6, 26 ----- 7-11, 27, 28
Y --- A	ORDENTLICH et al. Unique forms of human and mouse nuclear receptor corepressor SMRT. Proc Nat Acad Sci (USA) 16 March 1999 Vol 96 No 6 Pages 2639-2644. Especially pg 2642 fig 3B	20-23, 25, 26 ----- 24, 27, 28
A	CASTRO et al. Evidence that molecular changes in cells occur before morphological alterations during the progression of breast ductal carcinoma [online] Breast Cancer Research 17 October 2008 [retrieved 27 October 2010] Vol 10 Page R87 Available on the internet: <http://breast-cancer-research.com/content/10/5/R87>. Especially abstract, pg 15 Supplemental File 1	4, 7-11, 24, 27, 28

☒ 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

27 January 2010 (27.01.2010)

Date of mailing of the international search report

25 MAR 2010

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/67722

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 12-19
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/67722

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	GERADTS et al. Differential Copy Number Aberrations in New Candidate Genes Associated with Early Breast Cancer Progression. United States and Canadian Academy of Pathology 2009 Annual Meeting Abstract. 9 March 2009 [online] [retrieved 27 January 2010] . Available on the internet: < http://www.abstracts2view.com/uscap09/view.php?nu=USCAP09L_174 >. Abstract only.	1-11, 20-28
A	PORTER et al. Molecular Markers in Ductal Carcinoma in Situ of the Breast. Mol Cancer Res March 2003 Vol 1 Pages 362-375.	1-11, 20-28