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(54) GENE EXPRESSION PROFILES TO PREDICT BREAST CANCER OUTCOMES

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CPC ***C12Q 1/6886*** (2013.01); *G16B 40/00* (2019.02); ***G16B 25/00*** (2019.02)

(57) **ABSTRACT**

Methods for classifying and for evaluating the prognosis of a subject having breast cancer are provided. The methods include prediction of breast cancer subtype using a supervised algorithm trained to stratify subjects on the basis of breast cancer intrinsic subtype. The prediction model is based on the gene expression profile of the intrinsic genes listed in Table 1. This prediction model can be used to accurately predict the intrinsic subtype of a subject diagnosed with or suspected of having breast cancer. Further provided are compositions and methods for predicting outcome or response to therapy of a subject diagnosed with or suspected of having breast cancer. These methods are useful for guiding or determining treatment options for a subject afflicted with breast cancer. Methods of the invention further include means for evaluating gene expression profiles, including microarrays and quantitative polymerase chain reaction assays, as well as kits comprising reagents for practicing the methods of the invention.

Specification includes a Sequence Listing.

FIG. 1A

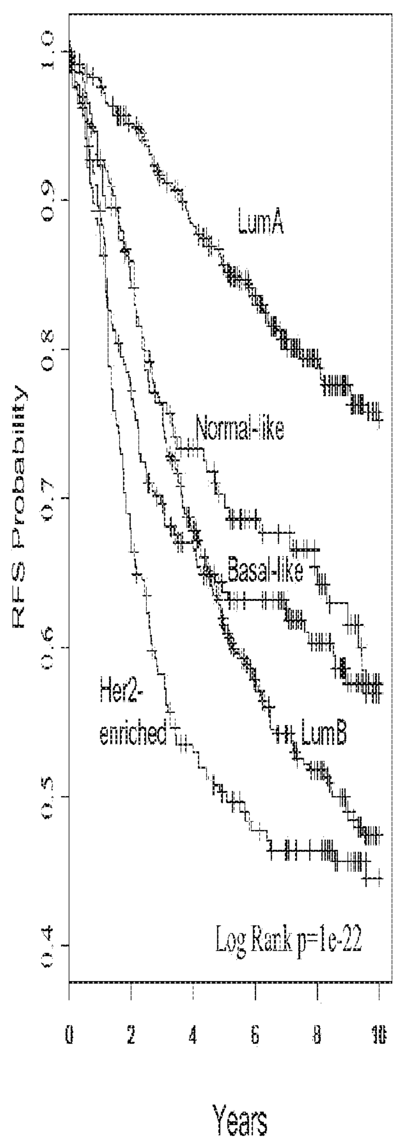

RFS Probability
1.0
0.9
0.8
0.7
0.6
0.5
0.4
LumA
Normal-like
Basal-like
Her2-
enriched
LumB
Log Rank p=1e-22
0
2
4
6
8
10
Years

FIG. 1B

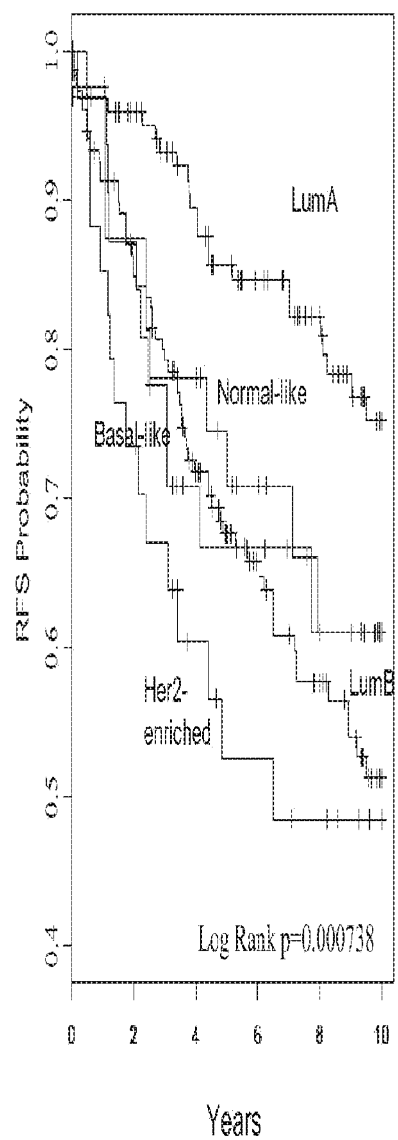

RFS Probability
1.0
0.9
0.8
0.7
0.6
0.5
0.4
LumA
Normal-like
Basal-like
Her2-
enriched
LumB
Log Rank p=0.000738
0
2
4
6
8
10
Years

FIG. 1C

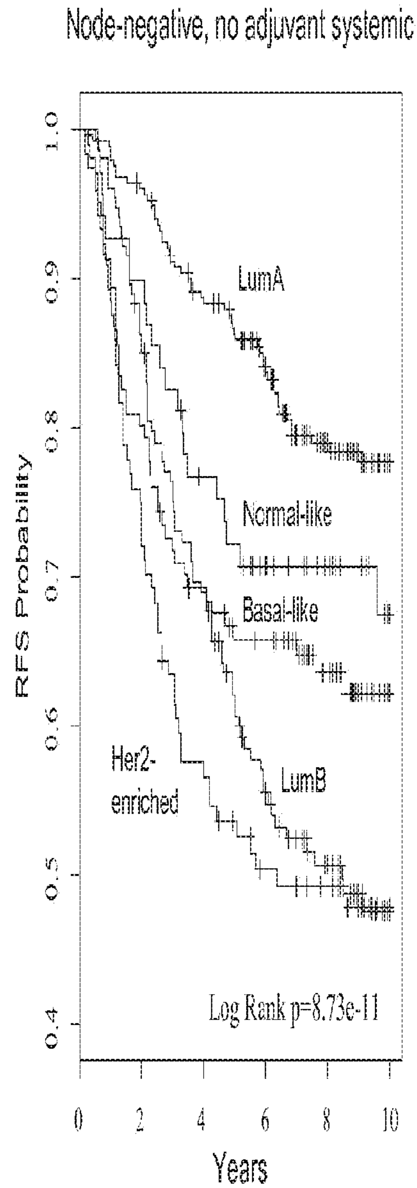

Node-negative, no adjuvant systemic
RFS Probability
1.0
0.9
0.8
0.7
0.6
0.5
0.4
LumA
Normal-like
Basal-like
LumB
Her2-
enriched
Log Rank p=8.73e-11
0
2
4
6
8
10
Years

FIG. 2A

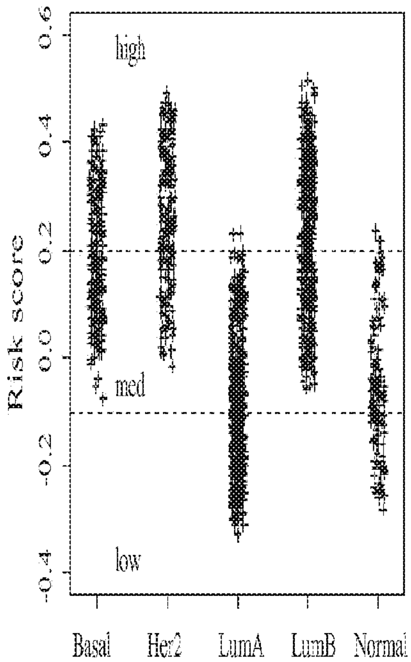


FIG. 2B

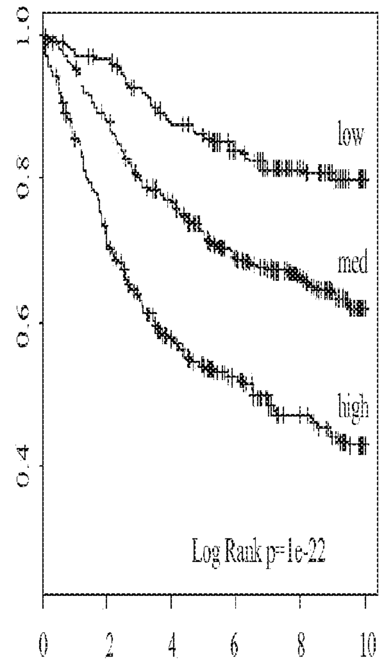


FIG. 2C

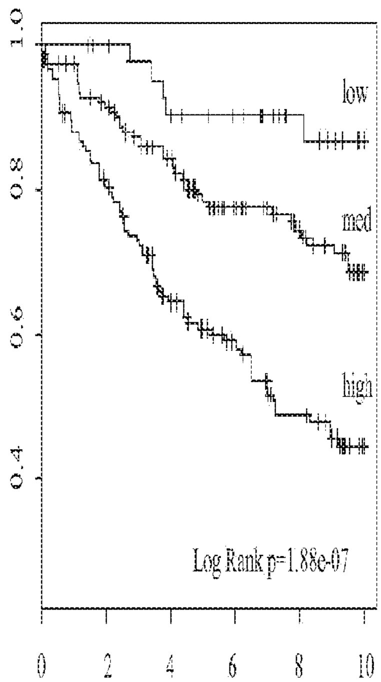


FIG. 2D

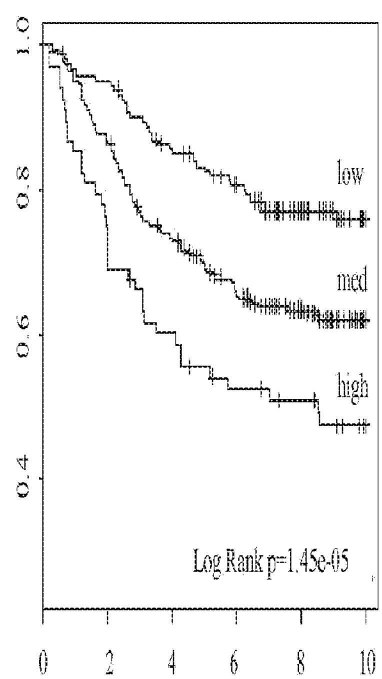


FIG. 3A

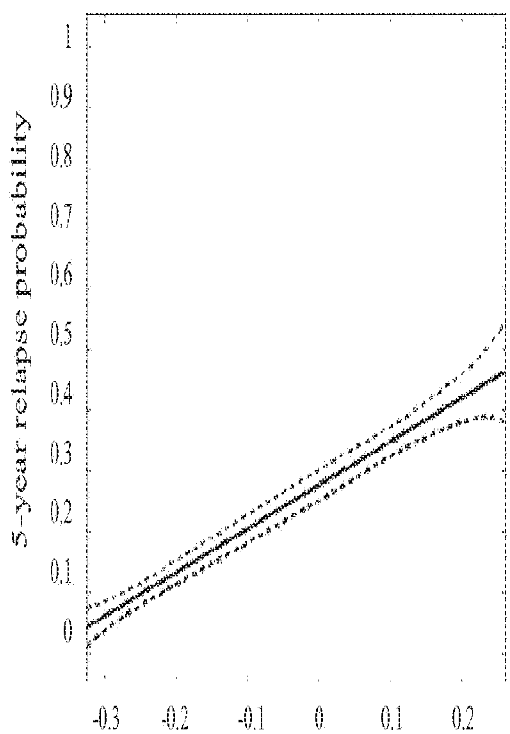

5-year relapse probability
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.3
-0.2
-0.1
0
0.1
0.2

FIG. 3B

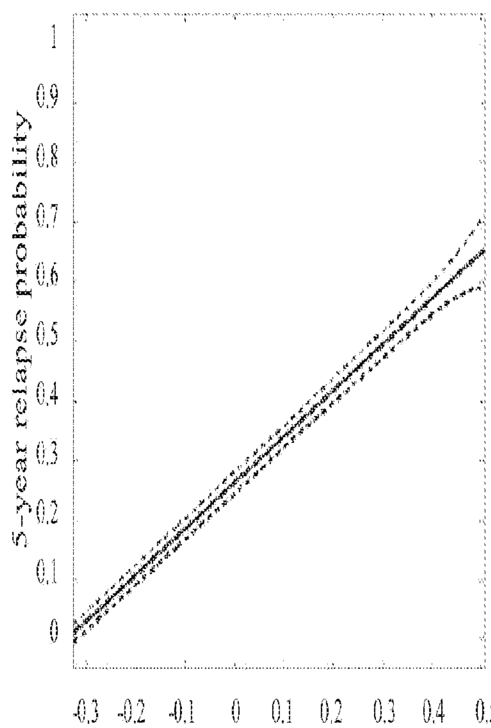

5-year relapse probability
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5

FIG. 4A

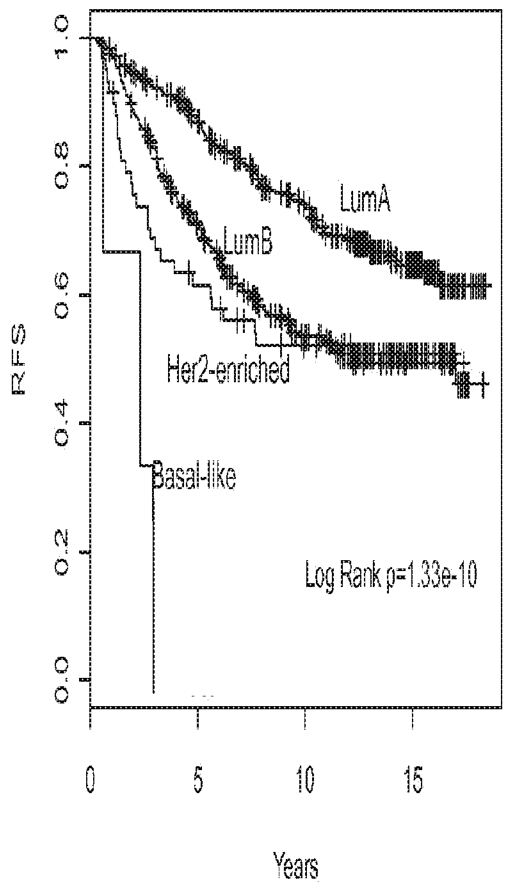

RFS
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Years
LumA
LumB
Her2-enriched
Basal-like
Log Rank p=1.33e-10

FIG. 4B

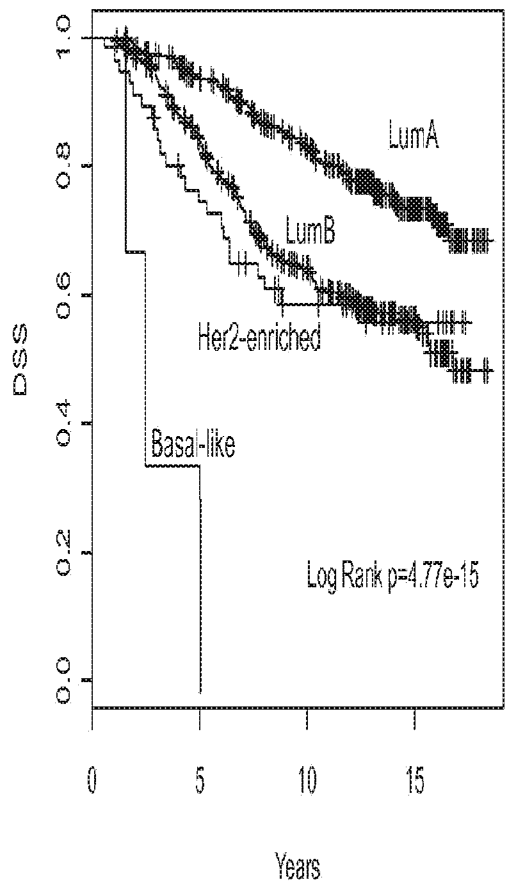
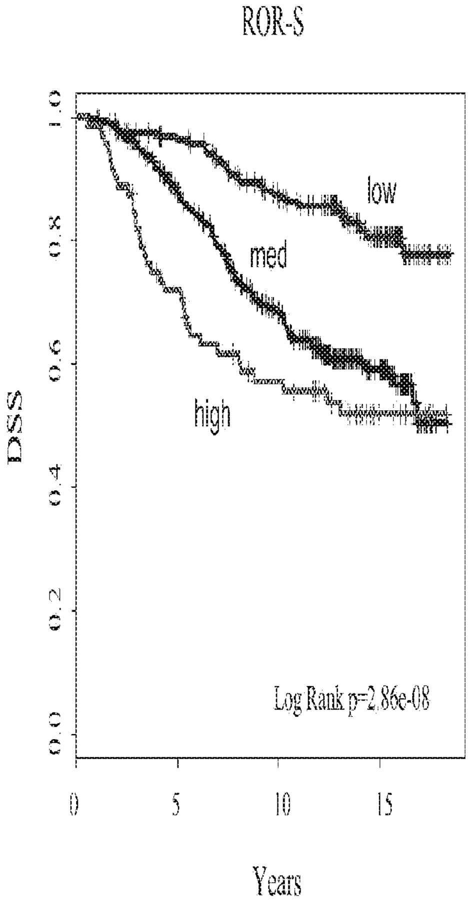
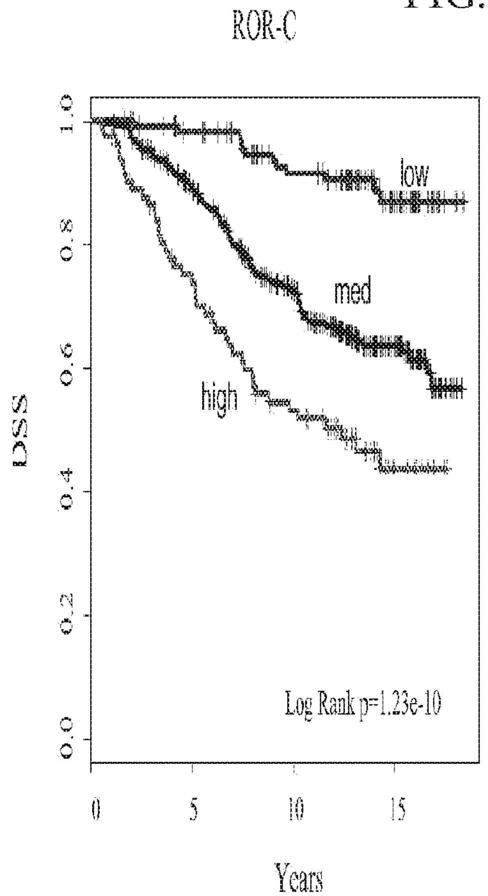

DSS
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Years
LumA
LumB
Her2-enriched
Basal-like
Log Rank p=4.77e-15

FIG. 5A



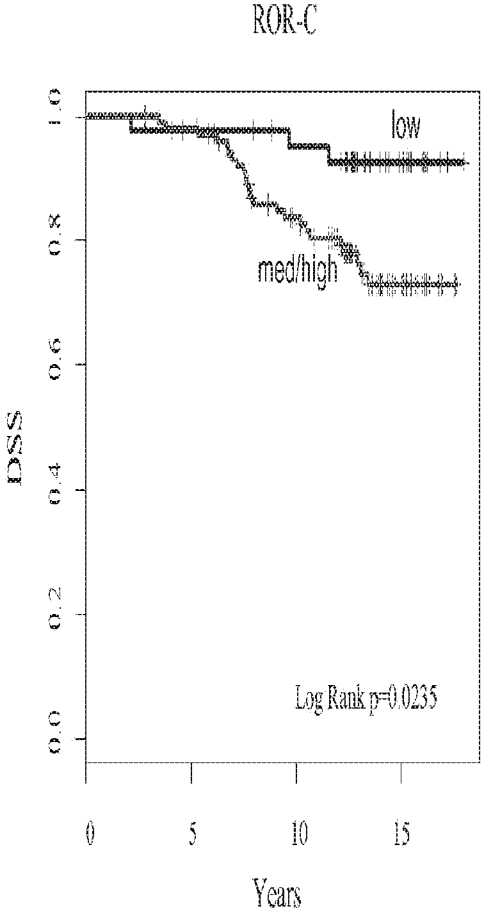
	10yr 95% CI	Events/N
Low	82-92%	31/193
Med	63-74%	117/330
High	45-69%	33/72

FIG. 5B



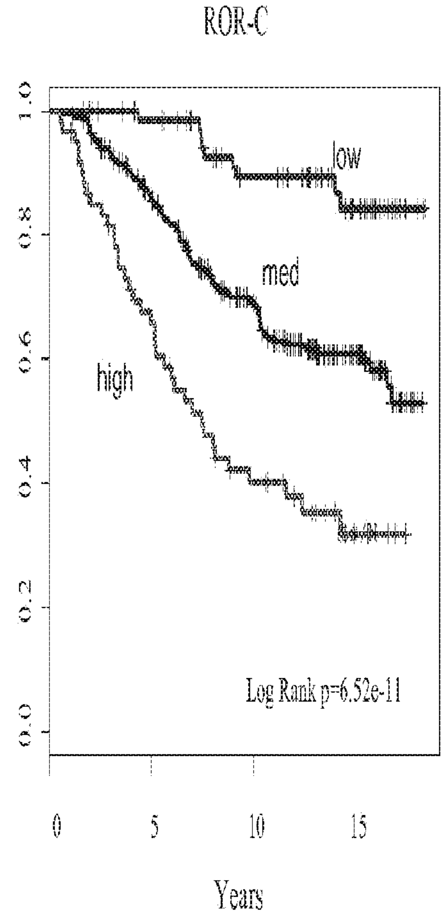
	10yr 95% CI	Events/N
Low	87-97%	12/124
Med	68-78%	108/338
High	43-65%	42/83

FIG. 6A



	10yr 95% CI	Events/N
Low	89-100%	3/43
Med/High	75-91%	24/105

FIG. 6B



	10yr 95% CI	Events/N
Low	82-97%	9/81
Med	63-75%	89/255
High	29-55%	37/61

FIG. 7A

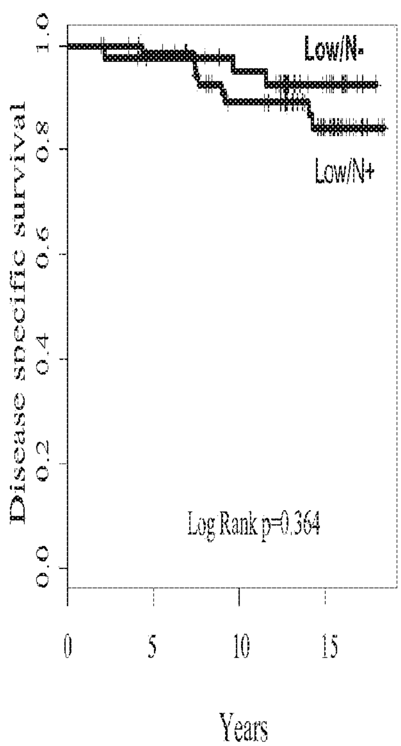
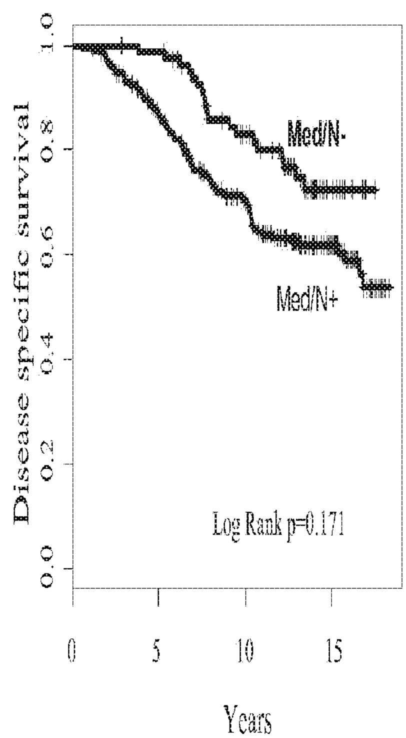


FIG. 7B



	10yr 95% CI	Events/N
Low/N-	89-100%	3/43
Low/N+	82-97%	9/80

	10yr 95% CI	Events/N
Med/N-	73-91%	19/83
Med/N+	64-76%	84/245

FIG. 7C

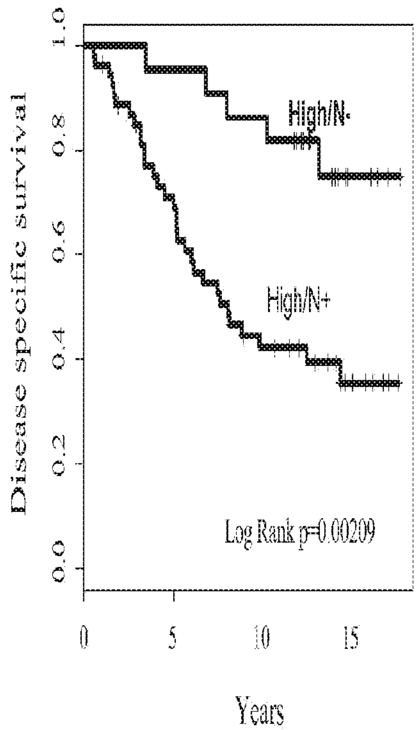
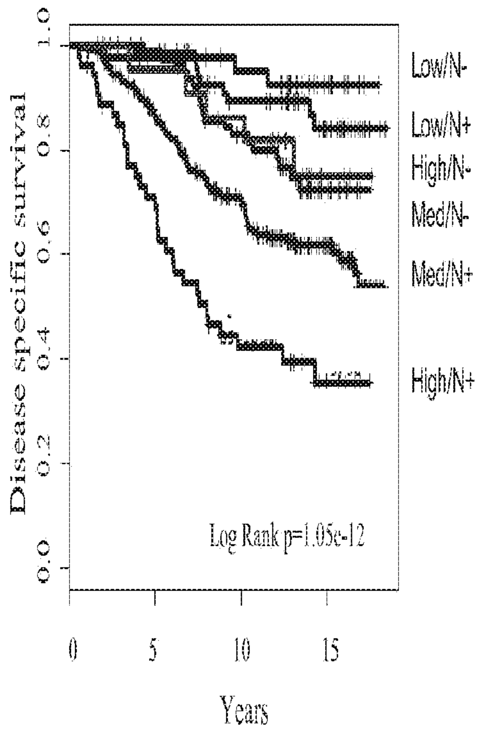


FIG. 7D



	10yr 95% CI	Events/N
High/N-	67-100%	5/22
High/N+	31-59%	31/54

	10yr 95% CI	Events/N
Low/N-	89-100%	3/43
Low/N+	82-97%	9/80
Med/N-	73-91%	19/83
Med/N+	64-76%	84/245
High/N-	67-100%	5/22
High/N+	31-59%	31/54

FIG. 8

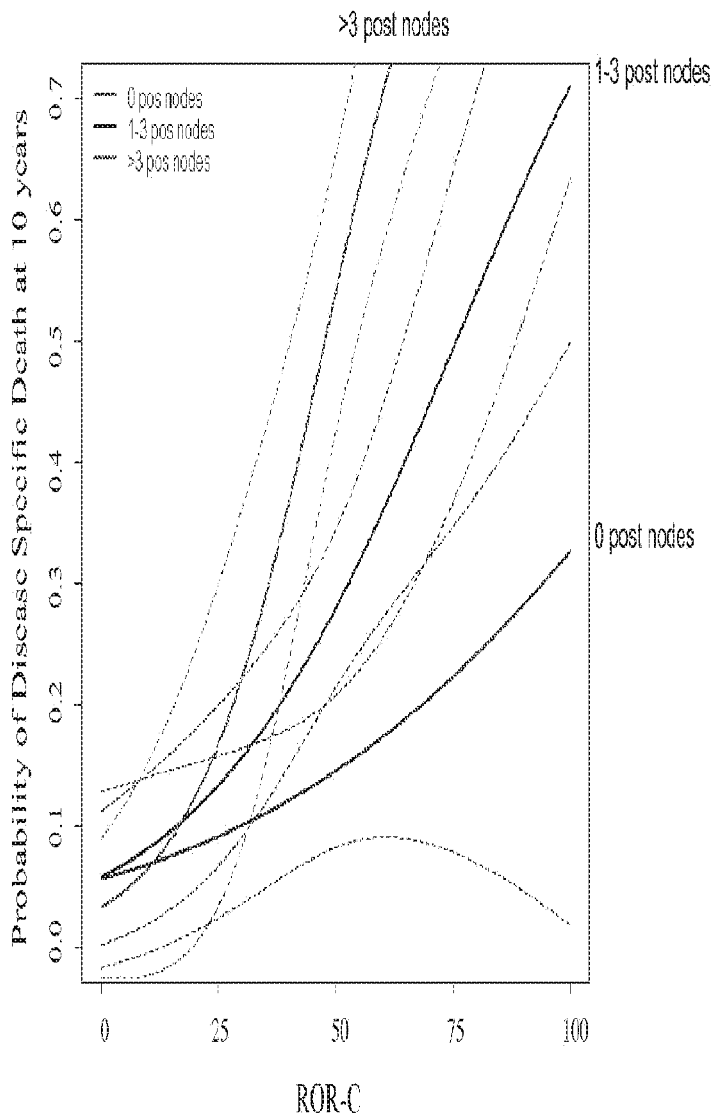

>3 post nodes
1-3 post nodes
0 post nodes
0 pos nodes
1-3 pos nodes
>3 pos nodes
Probability of Disease Specific Death at 10 years
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
25
50
75
100
ROR-C

FIG. 9

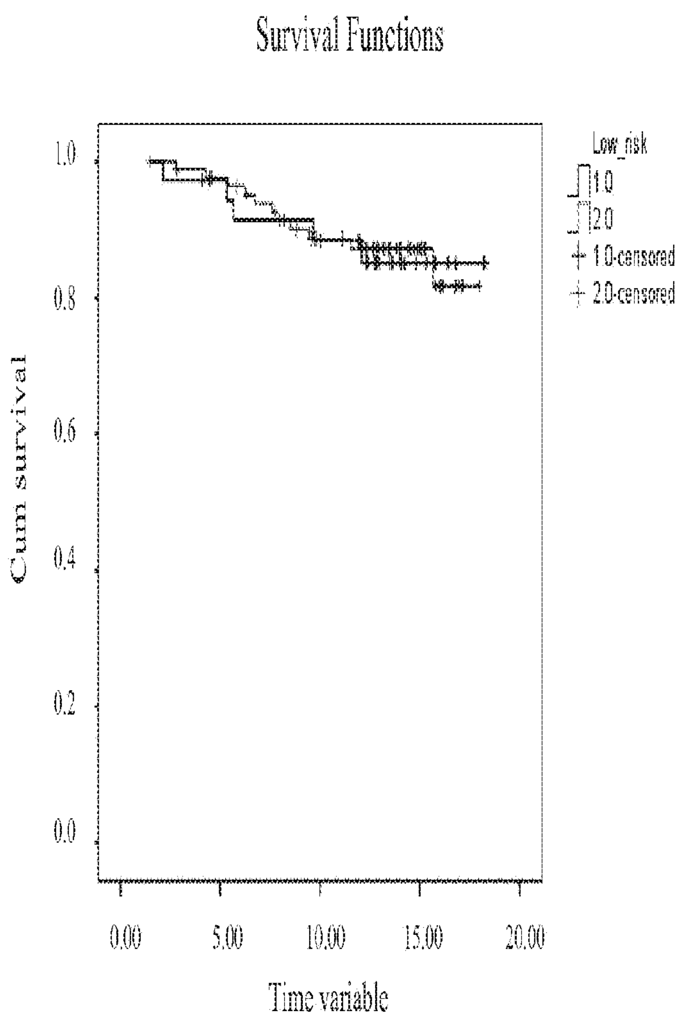

Survival Functions
Low_risk
1.0
2.0
1.0-censored
2.0-censored
1.0
0.8
0.6
0.4
0.2
0.0
Cum survival
0.00
5.00
10.00
15.00
20.00
Time variable

FIG. 10A

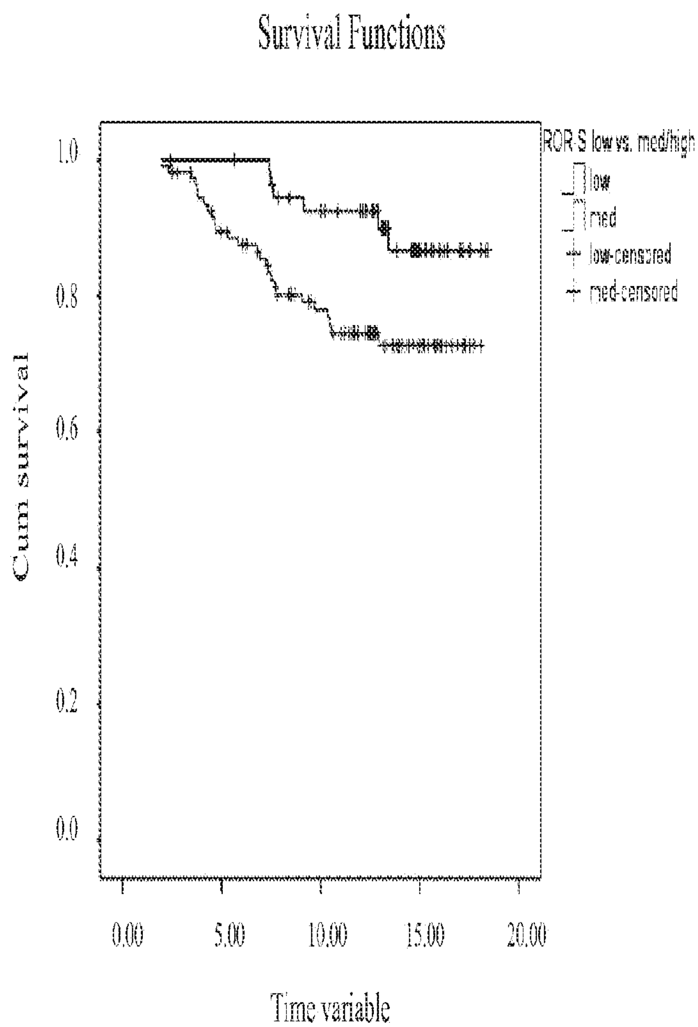

Survival Functions
ROR-S low vs. med/high
low
med
low-censored
med-censored
Cum survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
5.00
10.00
15.00
20.00
Time variable

FIG. 10B

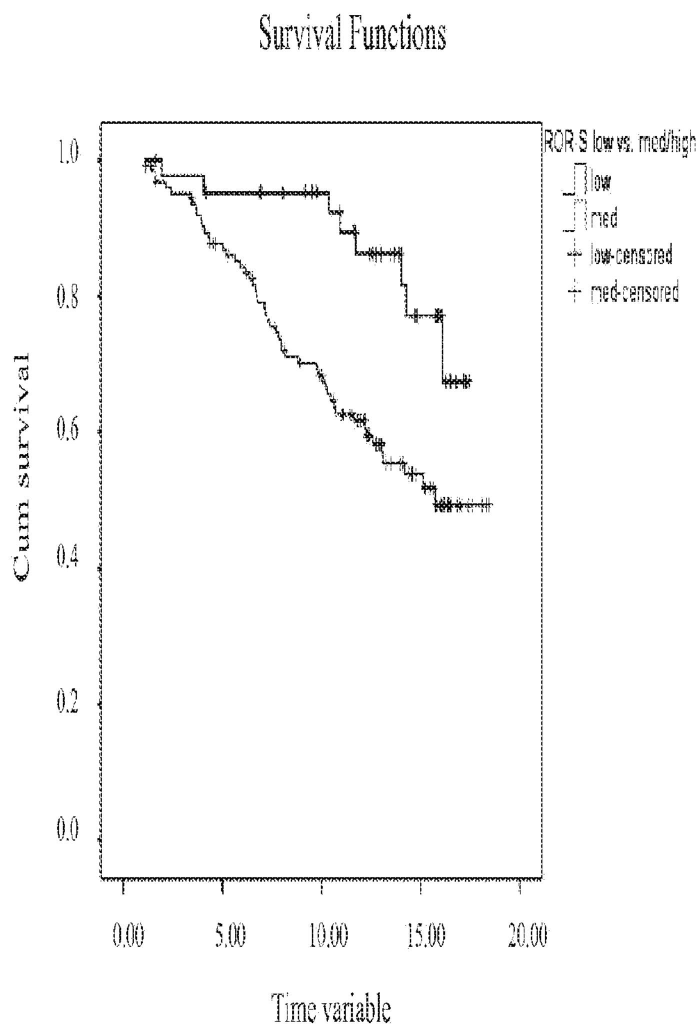
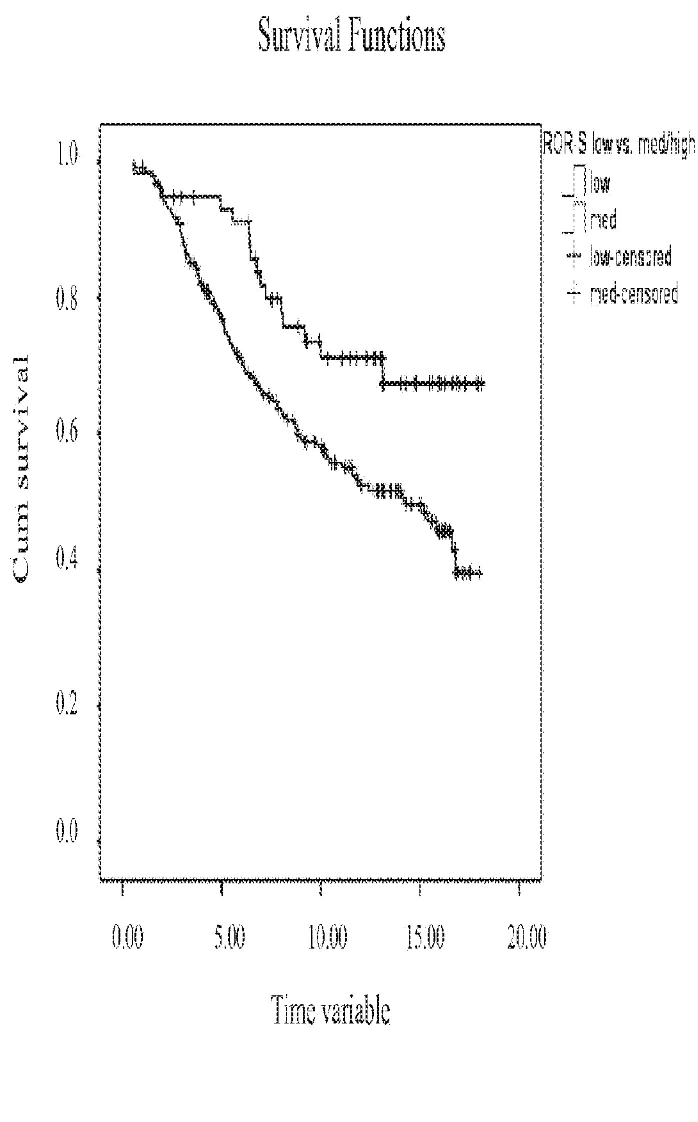

Survival Functions
ROR S low vs. med/high
low
med
low-censored
med-censored
Cum survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
5.00
10.00
15.00
20.00
Time variable

FIG. 10C


Survival Functions
Cum survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
5.00
10.00
15.00
20.00
Time variable
ROR-S low vs. med/high
low
med
low-censored
med-censored

GENE EXPRESSION PROFILES TO PREDICT BREAST CANCER OUTCOMES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. patent application Ser. No. 14/931,594 filed Nov. 3, 2015. U.S. patent application Ser. No. 14/931,594 is a divisional of U.S. patent application Ser. No. 12/995,450, filed Nov. 30, 2010, now U.S. Pat. No. 9,631,239. U.S. patent application Ser. No. 12/995,450 is a National Stage Application, filed under 35 U.S.C. § 371 of International Application No. PCT/US2009/045820, filed Jun. 1, 2009, which claims priority under 35 U.S.C. § 119 to U.S. Provisional Application Ser. No. 61/057,508, filed May 30, 2008. The contents of the aforementioned applications are incorporated herein by reference in their entireties.

GOVERNMENT INTEREST

[0002] This invention was made with government support under grant numbers R01 CA095614, U01 CA114722, and P50 CA582230 awarded by The National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Oct. 9, 2019, is named "NATE-702_C02US_SeqList.txt" and is 19.8 KB in size.

FIELD OF THE INVENTION

[0004] The present invention relates to methods for classifying breast cancer specimens into subtypes and for evaluating prognosis and response to therapy for patients afflicted with breast cancer.

BACKGROUND OF THE INVENTION

[0005] Breast cancer is the second most common cancer among women in the United States, second only to skin cancer. A woman in the U.S. has a one in eight chance of developing breast cancer during her lifetime, and the American Cancer Society estimates that more than 178,480 new cases of invasive breast cancer will be reported in the U.S. in 2007. Breast cancer is the second leading cause of cancer deaths in women, with more than 40,000 deaths annually. Improved detection methods, mass screening, and advances in treatment over the last decade have significantly improved the outlook for women diagnosed with breast cancer. Today, approximately 80% of breast cancer cases are diagnosed in the early stages of the disease when survival rates are at their highest. As a result, about 85% percent of breast cancer patients are alive at least five years after diagnosis. Despite these advances, approximately 20% of women diagnosed with early-stage breast cancer have a poor ten-year outcome and will suffer disease recurrence, metastasis or death within this time period.

[0006] Significant research has focused on identifying methods and factors for assessing breast cancer prognosis and predicting therapeutic response (See generally, Ross and Hortobagyi, eds. (2005) Molecular Oncology of Breast Cancer (Jones and Bartlett Publishers, Boston, Mass.) and the references cited therein). Prognostic indicators include conventional factors, such as tumor size, nodal status and histological grade, as well as molecular markers that provide some information regarding prognosis and likely response to particular treatments. For example, determination of estrogen (ER) and progesterone (PgR) steroid hormone receptor status has become a routine procedure in assessment of breast cancer patients. See, for example, Fitzgibbons et al., Arch. Pathol. Lab. Med. 124:966-78, 2000. Tumors that are hormone receptor positive are more likely to respond to hormone therapy and also typically grow less aggressively, thereby resulting in a better prognosis for patients with ER+/PgR+ tumors. Overexpression of human epidermal growth factor receptor 2 (HER-2/neu), a transmembrane tyrosine kinase receptor protein, has been correlated with poor breast cancer prognosis (see, e.g., Ross et al., The Oncologist 8:307-25, 2003), and HER-2 expression levels in breast tumors are used to predict response to the anti-HER-2 monoclonal antibody therapeutic trastuzumab (Herceptin®, Genentech, South San Francisco, Calif.).

SUMMARY OF THE INVENTION

[0007] Methods for classifying and for evaluating prognosis and treatment of a subject with breast cancer are provided. The methods include prediction of breast cancer subtype using a supervised algorithm trained to stratify subjects on the basis of breast cancer intrinsic subtype. The prediction model is based on the gene expression profile of the intrinsic genes listed in Table 1. In some embodiments, the algorithm is a nearest centroid algorithm, similar to the Prediction Analysis of Microarray (PAM) algorithm. The algorithm can be trained based on data obtained from the gene expression profiles deposited as accession number GSE10886 in the National Center for Biotechnology Information Gene Expression Omnibus. This prediction model, herein referred to as the PAM50 classification model, can be used to accurately predict the intrinsic subtype of a subject diagnosed with or suspected of having breast cancer.

[0008] Further provided are compositions and methods for predicting outcome or response to therapy of a subject diagnosed with or suspected of having breast cancer. These methods are useful for guiding or determining treatment options for a subject afflicted with breast cancer. Methods of the invention further include means for evaluating gene expression profiles, including microarrays and quantitative polymerase chain reaction assays, as well as kits comprising reagents for practicing the methods of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] FIGS. **1**A to **1**C shows outcomes based on subtype predictions using the PAM50 classifier. The PAM50 classification for LumA, LumB, HER2-enriched, Basal-like, and Normal-like shows prognostic significance for 1451 patients across all 5 test sets combined (FIG. **1**A), in 376 patients given endocrine therapy alone (FIG. **1**B), and in 701 node negative patients given no adjuvant systemic therapy (FIG. **1**C).

[0010] FIGS. **2**A to **2**D shows risk classification for test cases using a full model of intrinsic subtypes and two clinical variables. (FIG. **2**A) risk of relapse scores plotted for each breast cancer subtype: low risk scores <-0.1, moderate risk scores between -0.1 and 0.2, and high risk scores ≥ 0.2. (FIG. **2**B) Kaplan-Meier plots and significance of the risk score for all 1286 test samples, (FIG. **2**C) 376 patients that received adjuvant endocrine therapy only, and (FIG. **2**D) 560 patients that were node-negative and received no adjuvant systemic therapy.

[0011] FIGS. **3**A and **3**B shows the linear score for prognosis using the subtype-clinical model for risk of relapse at 5 years. Linear fit with 95% confidence intervals calibrates the risk of relapse score. The continuous risk model with subtype and clinical variables (T and N) was calibrated from 657 patients with ER-positive early stage breast cancer (FIG. **3**A), and in 1286 patients with ER-positive and ER-negative disease and stage 1-3 (FIG. **3**B).

[0012] FIGS. **4**A and **4**B shows association of PAM50 intrinsic subtype, determined by qPCR from paraffin blocks, with (FIG. **4**A) relapse free survival and (FIG. **4**B) disease-specific survival among 702 women with invasive breast carcinoma treated with adjuvant tamoxifen.

[0013] FIGS. **5**A and **5**B shows Kaplan-Meier analysis of breast cancer disease-specific survival for patients stratified into low, medium and high risk categories by applying the Risk-Of-Relapse algorithm to qPCR data generated from paraffin blocks. (FIG. **5**A) ROR-S, (FIG. **5**B) ROR-C.

[0014] FIGS. **6**A and **6**B shows Kaplan-Meier analysis of breast cancer disease-specific survival for patients stratified into low, medium and high risk categories (as defined previously on independent material by applying the ROR-C algorithm to women with (FIG. **6**A) node negative disease, and (FIG. **6**B) node positive disease).

[0015] FIGS. **7**A to **7**D shows Kaplan-Meier analysis of breast cancer disease-specific survival for patients stratified into node negative or node positive categories among women with a low risk ROR-C (FIG. **7**A), women with moderate risk ROR-C (FIG. **7**B); women with high risk ROR-C (FIG. **7**C). For direct comparison, all curves are superimposed in the lower right panel. Among women with low ROR-C, there is no significant difference in outcome by nodal status (FIG. **7**D).

[0016] FIG. **8** shows that an analysis of the ROR-C model versus probability of survival, stratified by the number of involved lymph nodes, reveals good outcomes regardless of nodal status category among patients with ROR-C values less than 25, who have overlapping 95% confidence intervals (denoted by dashed lines)

[0017] FIG. **9** shows the results of Kaplan-Meier analysis that was performed separately on each Adjuvant risk group, and differences in survival between the 90-95% and the 95-100% risk groups were tested using the log-rank test.

[0018] FIGS. **10**A to **10**C shows the results of Kaplan-Meier analysis that was performed separately on each Adjuvant risk group, and differences in survival between (FIG. **10**A) the Adjuvant Predicted BCSS 80-90%, ROR-S Low vs. Med/High; (FIG. **10**B) the Adjuvant Predicted BCSS 70-80%, ROR-S Low vs. Med/High; and, (FIG. **10**C) Adjuvant Predicted BCSS <70%, ROR-S Low vs. Med/High.

DETAILED DESCRIPTION OF THE INVENTION

[0019] Overview

[0020] Despite recent advances, the challenge of cancer treatment remains to target specific treatment regimens to distinct tumor types with different pathogenesis, and ultimately personalize tumor treatment in order to maximize outcome. In particular, once a patient is diagnosed with cancer, such as breast cancer, there is a need for methods that allow the physician to predict the expected course of disease, including the likelihood of cancer recurrence, long-term survival of the patient and the like, and select the most appropriate treatment options accordingly.

[0021] For the purposes of the present invention, "breast cancer" includes, for example, those conditions classified by biopsy or histology as malignant pathology. The clinical delineation of breast cancer diagnoses is well-known in the medical arts. One of skill in the art will appreciate that breast cancer refers to any malignancy of the breast tissue, including, for example, carcinomas and sarcomas. Particular embodiments of breast cancer include ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS), or mucinous carcinoma. Breast cancer also refers to infiltrating ductal (IDC) or infiltrating lobular carcinoma (ILC). In most embodiments of the invention, the subject of interest is a human patient suspected of or actually diagnosed with breast cancer.

[0022] Breast cancer is a heterogeneous disease with respect to molecular alterations and cellular composition. This diversity creates a challenge for researchers trying to develop classifications that are clinically meaningful. Gene expression profiling by microarray has provided insight into the complexity of breast tumors and can be used to provide prognostic information beyond standard pathologic parameters (1-7).

[0023] Expression profiling of breast cancer identifies biologically and clinically distinct molecular subtypes which may require different treatment approaches [van't Veer 2005][Loi 2007][Cheang 2008a]. The major intrinsic subtypes of breast cancer referred to as Luminal A, Luminal B, HER2-enriched, Basal-like have distinct clinical features, relapse risk and response to treatment [Sorlie 2003]. The "intrinsic" subtypes known as Luminal A (LumA), Luminal B (LumB), HER2-enriched, Basal-like, and Normal-like were discovered using unsupervised hierarchical clustering of microarray data (1, 8). Intrinsic genes, as described in Perou et al. (2000) Nature 406:747-752, are statistically selected to have low variation in expression between biological sample replicates from the same individual and high variation in expression across samples from different individuals. Thus, intrinsic genes are the classifier genes for breast cancer classification. Although clinical information was not used to derive the breast cancer intrinsic subtypes, this classification has proved to have prognostic significance (1, 6, 9, 10).

[0024] Breast tumors of the "Luminal" subtype are ER positive and have a similar keratin expression profile as the epithelial cells lining the lumen of the breast ducts (Taylor-Papadimitriou et al. (1989) J Cell Sci 94:403-413; Perou et al (2000) New Technologies for Life Sciences: A Trends Guide 67-76, each of which is herein incorporated by reference in its entirety). Conversely, ER-negative tumors can be broken into two main subtypes, namely those that overexpress (and are DNA amplified for) HER-2 and GRB7 (HER-2-enriched) and "Basal-like" tumors that have an expression profile similar to basal epithelium and express Keratin 5, 6B, and 17. Both these tumor subtypes are aggressive and typically more deadly than Luminal tumors; however, there are subtypes of Luminal tumors with different outcomes. The Luminal tumors with poor outcomes consistently share the histopathological feature of being higher grade and the molecular feature of highly expressing proliferation genes.

[0025] The translation of the intrinsic subtypes into a clinical assay has been challenging because unsupervised clustering is better suited to organizing large numbers of samples and genes than classifying individual samples using small gene sets.

[0026] Thus, provided herein are improved methods and compositions for classifying breast cancer intrinsic subtypes. The methods utilize a supervised algorithm to classify subject samples according to breast cancer intrinsic subtype. This algorithm, referred to herein as the PAM50 classification model, is based on the gene expression profile of a defined subset of intrinsic genes that has been identified herein as superior for classifying breast cancer intrinsic subtypes, and for predicting risk of relapse and/or response to therapy in a subject diagnosed with breast cancer. The subset of genes, along with primers specific for their detection, is provided in Table 1.

[0027] In some embodiments, at least about 40 of the genes listed in Table 1 are used in the PAM50 classification model. In other embodiments, at least 41, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, or all 50 of the intrinsic genes listed in Table 1 are used in the model. The methods disclosed herein are not intended for use with one or only a few of the genes listed in Table 1. In fact, it is the combination of substantially all of the intrinsic genes that allows for the most accurate classification of intrinsic subtype and prognostication of outcome or therapeutic response to treatment. Thus, in various embodiments, the methods disclosed herein encompass obtaining the genetic profile of substantially all the genes listed in Table 1. "Substantially all" may encompass at least 47, at least 48, at least 49, or all 50 of the genes listed in Table 1. Unless otherwise specified, "substantially all" refers to at least 49 of the genes listed in Table 1. It will also be understood by one of skill in the art that one subset of the genes listed in Table 1 can be used to train an algorithm to predict breast cancer subtype or outcome, and another subset of the genes used to characterize an individual subject. Preferably, all 50 genes are used to train the algorithm, and at least 49 of the genes are used to characterize a subject.

TABLE 1

PAM50 Intrinsic Gene List

GENE NAME	REPRESENTATIVE GENBANK ACCESSION NUMBER	FORWARD PRIMER	SEQ ID NO:	REVERSE PRIMER	SEQ ID NO:
ACTR3B	NM_020445NM_001040135	AAAGATTCCTGGGACCTGA	1	TGGGGCAGTTCTGTATTACTTC	51
ANLN	NM_018685	ACAGCCACTTTCAGAAGCAAG	2	CGATGGTTTTGTACAAGATTTCTC	52
BAG1	NM_004323	CTGGAAGAGTTGAATAAAGAGC	3	GCAAATCCTTGGGCAGA	53
BCL2	NM_000633	TACCTGAACCGGCACCTG	4	GCCGTACAGTTCCACAAAGG	54
BIRC5	NM_001012271	GCACAAAGCCATTCTAAGTC	5	GACGCTTCCTATCACTCTATTC	55
BLVRA	BX647539	GCTGGCTGAGCAGAAAG	6	TTCCTCCATCAAGAGTTCAACA	56
CCNB1	NM_031966	CTTTCGCCTGAGCCTATTT	7	GGGCACATCCAGATGTTT	57
CCNE1	BC035498	GGCCAAAATCGACAGGAC	8	GGGTCTGCACAGACTGCAT	58
CDC20	BG256659	CTGTCTGAGTGCCGTGGAT	9	TCCTTGTAATGGGGAGACCA	59
CDC6	NM_001254	GTAAATCACCTTCTGAGCCT	10	ACTTGGGATATGTGAATAAGACC	60
CDCA1	NM_031423	GGAGGCGGAAGAAACCAG	11	GGGGAAAGACAAAGTTTCCA	61
CDH3	BC041846	GACAAGGAGAATCAAAAGATCAGC	12	ACTGTCTGGGTCCATGGCTA	62
CENPF	NM_016343	GTGGCAGCAGATCACAA	13	GGATTTCGTGGTGGGTTC	63
CEP55	AB091343	CCTCACGAATTGCTGAACTT	14	CCACAGTCTGTGATAAACGG	64

TABLE 1-continued

PAM50 Intrinsic Gene List

GENE NAME	REPRESENTATIVE GENBANK ACCESSION NUMBER	FORWARD PRIMER	SEQ ID NO:	REVERSE PRIMER	SEQ ID NO:
CXXC5	BC006428	CATGAAATAGTG CATAGTTTGCC	15	CCATCAACATTCT CTTTATGAACG	65
EGFR	NM_005228	ACACAGAATCTAT ACCCACCAGAGT	16	ATCAACTCCCAAA CGGTCAC	66
ERBB2	NM_001005862	GCTGGCTCTCAC ACTGATAG	17	GCCCTTACACATC GGAGAAC	67
ESR1	NM_001122742	GCAGGGAGAGGA GTTTGT	18	GACTTCAGGGTGC TGGAC	68
EXO1	NM_130398	CCCATCCATGTG AGGAAGTATAA	19	TGTGAAGCCAGCA ATATGTATC	69
FGFR4	AB209631	CTTCTTGGACCTT GGCG	20	TATTGGGAGGCAG GAGGTTTA	70
FOXA1	NM_004496	GCTACTACGCAG ACACG	21	CTGAGTTCATGTT GCTGACC	71
FOXC1	NM_001453	GATGTTCGAGTC ACAGAGG	22	GACAGCTACTATT CCCGTT	72
GPR160	AJ249248	TTCGGCTGGAAG GAACC	23	TATGTGAGTAAGC TCGGAGAC	73
GRB7	NM_005310	CGTGGCAGATGT GAACGA	24	AGTGGGCATCCCG TAGA	74
HSPC150 (UBE2T)	NM_014176	GGAGATCCGTCA ACTCCAAA	25	AGTGGACATGCGA GTGGAG	75
KIF2C	NM_006845	TGGGTCGTGTCA GGAAAC	26	CACCGCTGGAAAC TGAAC	76
KNTC2	NM_006101	CGCAGTCATCCA GAGATGTG	27	CGTGCACATCCAT GACCTT	77
KRT14	BC042437	ACTCAGTACAAG AAAGAACCG	28	GAGGAGATGACCT TGCC	78
KRT17	AK095281	GTTGGACCAGTC AACATCTCTG	29	GCCATAGCCACTG CCACT	79
KRT5	M21389	TGTGGCTCATTA GGCAAC	30	CTTCGACTGGACT CTGT	80
MAPT	NM_001123066	GACTCCAAGCGC GAAAAC	31	CAGACATGTTGGT ATTGCACATT	81
MDM2	M92424	CCACAAAATATT CATGGTTCTTG	32	AGGCGATCCTGGG AAATTAT	82
MELK	NM_014791	CCAGTAGCATTG TCCGAG	33	CCCATTTGTCTGTC TTCAC	83
MIA	BG765502	GTCTCTGGTAAT GCACACT	34	CTGATGGTTGAGG CTGTT	84
MKI67	NM_002417	GTGGAATGCCTG CTGACC	35	CGCACTCCAGCAC CTAGAC	85
MLPH	NM_024101	AGGGGTGCCCTC TGAGAT	36	TCACAGGGTCAAA CTTCCAGT	86
MMP11	NM_005940	CGAGATCGCCAA GATGTT	37	GATGGTAGAGTTC CAGTGATT	87

TABLE 1-continued

PAM50 Intrinsic Gene List

GENE NAME	REPRESENTATIVE GENBANK ACCESSION NUMBER	FORWARD PRIMER	SEQ ID NO:	REVERSE PRIMER	SEQ ID NO:
MYBL2	BX647151	AGGCGAACACAC AACGTC	38	TCTGGTCACGCAG GGCAA	88
MYC	NM_002467	AGCCTCGAACAA TTGAAGA	39	ACACAGATGATGG AGATGTC	89
NAT1	BC013732	ATCGACTGTGTAAA CAACTAGAGAAGA	40	AGTAGCTACATCT CCAGGTTCTCTG	90
ORC6L	NM_014321	TTTAAGAGGGCA AATGGAAGG	41	CGGATTTTATCAA CGATGCAG	91
PGR	NM_000926	TGCCGCAGAACT CACTTG	42	CATTTGCCGTCCTT CATCG	92
PHGDH	AK093306	CCTCAGATGATG CCTATCCA	43	GCAGGTCAAAACT CTCAAAG	93
PTTG1	BE904476	CAGCAAGCGATG GCATAGT	44	AGCGGGCTTCTGT AATCTGA	94
RRM2	AK123010	AATGCCACCGAA GCCTC	45	GCCTCAGATTTCA ACTCGT	95
SFRP1	BC036503	TCGAACTGAAGG CTATTTACGAG	46	CTGCTGAGAATCA AAGTGGGA	96
SLC39A6	NM_012319	GTCGAAGCCGCA ATTAGG	47	GGAACAAACTGCT CTGCCA	97
TMEM45 B	AK098106	CAAACGTGTGTT CTGGAGG	48	ACAGCTCTTTAGC ATTTGTGGA	98
TYMS	BQ56428	TGCCCTGTATGAT GTCAGGA	49	GGGACTATCAATG TTGGGTTCTC	99
UBE2C	BC032677	GTGAGGGGTGTC AGCTCAGT	50	CACACAGTTCACT GCTCCACA	100

[0028] "Gene expression" as used herein refers to the relative levels of expression and/or pattern of expression of a gene. The expression of a gene may be measured at the level of DNA, cDNA, RNA, mRNA, or combinations thereof "Gene expression profile" refers to the levels of expression of multiple different genes measured for the same sample. An expression profile can be derived from a biological sample collected from a subject at one or more time points prior to, during, or following diagnosis, treatment, or therapy for breast cancer (or any combination thereof), can be derived from a biological sample collected from a subject at one or more time points during which there is no treatment or therapy for breast cancer (e.g., to monitor progression of disease or to assess development of disease in a subject at risk for breast cancer), or can be collected from a healthy subject. Gene expression profiles may be measured in a sample, such as samples comprising a variety of cell types, different tissues, different organs, or fluids (e.g., blood, urine, spinal fluid, sweat, saliva or serum) by various methods including but not limited to microarray technologies and quantitative and semi-quantitative RT-PCR techniques.

Clinical Variables

[0029] The PAM50 classification model described herein may be further combined with information on clinical variables to generate a continuous risk of relapse (ROR) predictor. As described herein, a number of clinical and prognostic breast cancer factors are known in the art and are used to predict treatment outcome and the likelihood of disease recurrence. Such factors include, for example, lymph node involvement, tumor size, histologic grade, estrogen and progesterone hormone receptor status, HER-2 levels, and tumor ploidy.

[0030] In one embodiment, risk of relapse (ROR) score is provided for a subject diagnosed with or suspected of having breast cancer. This score uses the PAM50 classification model in combination with clinical factors of lymph node status (N) and tumor size (T). Assessment of clinical variables is based on the American Joint Committee on Cancer (AJCC) standardized system for breast cancer staging. In this system, primary tumor size is categorized on a scale of 0-4 (T0: no evidence of primary tumor; T1: ≤2 cm; T2: >2 cm-≤5 cm; T3: >5 cm; T4: tumor of any size with direct spread to chest wall or skin). Lymph node status is classified as N0-N3 (N0: regional lymph nodes are free of metastasis; N1: metastasis to movable, same-side axillary lymph node (s); N2: metastasis to same-side lymph node(s) fixed to one another or to other structures; N3: metastasis to same-side lymph nodes beneath the breastbone). Methods of identifying breast cancer patients and staging the disease are well known and may include manual examination, biopsy, review of patient's and/or family history, and imaging techniques, such as mammography, magnetic resonance imaging (MM), and positron emission tomography (PET).

[0031] Using the PAM50 classification methods of the present invention, the prognosis of a breast cancer patient can be determined independent of or in combination with assessment of these clinical factors. In some embodiments, combining the PAM50 breast cancer intrinsic subtype classification methods disclosed herein with evaluation of these clinical factors may permit a more accurate risk assessment. The methods of the invention may be further coupled with analysis of, for example, estrogen receptor (ER) and progesterone receptor (PgR) status, and/or HER-2 expression levels. Other factors, such as patient clinical history, family history and menopausal status, may also be considered when evaluating breast cancer prognosis via the methods of the invention.

Sample Source

[0032] In one embodiment of the present invention, breast cancer subtype is assessed through the evaluation of expression patterns, or profiles, of the intrinsic genes listed in Table 1 in one or more subject samples. For the purpose of discussion, the term subject, or subject sample, refers to an individual regardless of health and/or disease status. A subject can be a subject, a study participant, a control subject, a screening subject, or any other class of individual from whom a sample is obtained and assessed in the context of the invention. Accordingly, a subject can be diagnosed with breast cancer, can present with one or more symptoms of breast cancer, or a predisposing factor, such as a family (genetic) or medical history (medical) factor, for breast cancer, can be undergoing treatment or therapy for breast cancer, or the like. Alternatively, a subject can be healthy with respect to any of the aforementioned factors or criteria. It will be appreciated that the term "healthy" as used herein, is relative to breast cancer status, as the term "healthy" cannot be defined to correspond to any absolute evaluation or status. Thus, an individual defined as healthy with reference to any specified disease or disease criterion, can in fact be diagnosed with any other one or more diseases, or exhibit any other one or more disease criterion, including one or more cancers other than breast cancer. However, the healthy controls are preferably free of any cancer.

[0033] In particular embodiments, the methods for predicting breast cancer intrinsic subtypes include collecting a biological sample comprising a cancer cell or tissue, such as a breast tissue sample or a primary breast tumor tissue sample. By "biological sample" is intended any sampling of cells, tissues, or bodily fluids in which expression of an intrinsic gene can be detected. Examples of such biological samples include, but are not limited to, biopsies and smears. Bodily fluids useful in the present invention include blood, lymph, urine, saliva, nipple aspirates, gynecological fluids, or any other bodily secretion or derivative thereof. Blood can include whole blood, plasma, serum, or any derivative of blood. In some embodiments, the biological sample includes breast cells, particularly breast tissue from a biopsy, such as a breast tumor tissue sample. Biological samples may be obtained from a subject by a variety of techniques including, for example, by scraping or swabbing an area, by using a needle to aspirate cells or bodily fluids, or by removing a tissue sample (i.e., biopsy). Methods for collecting various biological samples are well known in the art. In some embodiments, a breast tissue sample is obtained by, for example, fine needle aspiration biopsy, core needle biopsy, or excisional biopsy. Fixative and staining solutions may be applied to the cells or tissues for preserving the specimen and for facilitating examination. Biological samples, particularly breast tissue samples, may be transferred to a glass slide for viewing under magnification. In one embodiment, the biological sample is a formalin-fixed, paraffin-embedded breast tissue sample, particularly a primary breast tumor sample. In various embodiments, the tissue sample is obtained from a pathologist-guided tissue core sample as described in Example 4.

Expression Profiling

[0034] In various embodiments, the present invention provides methods for classifying, prognosticating, or monitoring breast cancer in subjects. In this embodiment, data obtained from analysis of intrinsic gene expression is evaluated using one or more pattern recognition algorithms. Such analysis methods may be used to form a predictive model, which can be used to classify test data. For example, one convenient and particularly effective method of classification employs multivariate statistical analysis modeling, first to form a model (a "predictive mathematical model") using data ("modeling data") from samples of known subtype (e.g., from subjects known to have a particular breast cancer intrinsic subtype. LumA, LumB, Basal-like, HER2-enriched, or normal-like), and second to classify an unknown sample (e.g., "test sample") according to subtype.

[0035] Pattern recognition methods have been used widely to characterize many different types of problems ranging, for example, over linguistics, fingerprinting, chemistry and psychology. In the context of the methods described herein, pattern recognition is the use of multivariate statistics, both parametric and non-parametric, to analyze data, and hence to classify samples and to predict the value of some dependent variable based on a range of observed measurements. There are two main approaches. One set of methods is termed "unsupervised" and these simply reduce data complexity in a rational way and also produce display plots which can be interpreted by the human eye. However, this type of approach may not be suitable for developing a clinical assay that can be used to classify samples derived from subjects independent of the initial sample population used to train the prediction algorithm.

[0036] The other approach is termed "supervised" whereby a training set of samples with known class or outcome is used to produce a mathematical model which is then evaluated with independent validation data sets. Here, a "training set" of intrinsic gene expression data is used to construct a statistical model that predicts correctly the "subtype" of each sample. This training set is then tested with independent data (referred to as a test or validation set) to determine the robustness of the computer-based model. These models are sometimes termed "expert systems," but may be based on a range of different mathematical procedures. Supervised methods can use a data set with reduced dimensionality (for example, the first few principal components), but typically use unreduced data, with all dimensionality. In all cases the methods allow the quantitative description of the multivariate boundaries that characterize and separate each subtype in terms of its intrinsic gene expression profile. It is also possible to obtain confidence limits on any predictions, for example, a level of probability to be placed on the goodness of fit (see, for example, Kowalski et al., 1986). The robustness of the predictive models can also be checked using cross-validation, by leaving out selected samples from the analysis.

[0037] The PAM50 classification model described herein is based on the gene expression profile for a plurality of subject samples using the intrinsic genes listed in Table 1. The plurality of samples includes a sufficient number of samples derived from subjects belonging to each subtype class. By "sufficient samples" or "representative number" in this context is intended a quantity of samples derived from each subtype that is sufficient for building a classification model that can reliably distinguish each subtype from all others in the group. A supervised prediction algorithm is developed based on the profiles of objectively-selected prototype samples for "training" the algorithm. The samples are selected and subtyped using an expanded intrinsic gene set according to the methods disclosed in International Patent Publication WO 2007/061876, which is herein incorporated by reference in its entirety. Alternatively, the samples can be subtyped according to any known assay for classifying breast cancer subtypes. After stratifying the training samples according to subtype, a centroid-based prediction algorithm is used to construct centroids based on the expression profile of the intrinsic gene set described in Table 1.

[0038] In one embodiment, the prediction algorithm is the nearest centroid methodology related to that described in Narashiman and Chu (2002) PNAS 99:6567-6572, which is herein incorporated by reference in its entirety. In the present invention, the method computes a standardized centroid for each subtype. This centroid is the average gene expression for each gene in each subtype (or "class") divided by the within-class standard deviation for that gene. Nearest centroid classification takes the gene expression profile of a new sample, and compares it to each of these class centroids. Subtype prediction is done by calculating the Spearman's rank correlation of each test case to the five centroids, and assigning a sample to a subtype based on the nearest centroid.

Detection of Intrinsic Gene Expression

[0039] Any methods available in the art for detecting expression of the intrinsic genes listed in Table 1 are encompassed herein. By "detecting expression" is intended determining the quantity or presence of an RNA transcript or its expression product of an intrinsic gene.

[0040] Methods for detecting expression of the intrinsic genes of the invention, that is, gene expression profiling, include methods based on hybridization analysis of polynucleotides, methods based on sequencing of polynucleotides, immunohistochemistry methods, and proteomics-based methods. The methods generally detect expression products (e.g., mRNA) of the intrinsic genes listed in Table 1. In preferred embodiments, PCR-based methods, such as reverse transcription PCR (RT-PCR) (Weis et al., TIG 8:263-64, 1992), and array-based methods such as microarray (Schena et al., Science 270:467-70, 1995) are used. By "microarray" is intended an ordered arrangement of hybridizable array elements, such as, for example, polynucleotide probes, on a substrate. The term "probe" refers to any molecule that is capable of selectively binding to a specifically intended target biomolecule, for example, a nucleotide transcript or a protein encoded by or corresponding to an intrinsic gene. Probes can be synthesized by one of skill in the art, or derived from appropriate biological preparations. Probes may be specifically designed to be labeled. Examples of molecules that can be utilized as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic molecules.

[0041] Many expression detection methods use isolated RNA. The starting material is typically total RNA isolated from a biological sample, such as a tumor or tumor cell line, and corresponding normal tissue or cell line, respectively. If the source of RNA is a primary tumor, RNA (e.g., mRNA) can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g., formalin-fixed) tissue samples (e.g., pathologist-guided tissue core samples).

[0042] General methods for RNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, New York 1987-1999. Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp and Locker (Lab Invest. 56:A67, 1987) and De Andres et al. (Biotechniques 18:42-44, 1995). In particular, RNA isolation can be performed using a purification kit, a buffer set and protease from commercial manufacturers, such as Qiagen (Valencia, Calif.), according to the manufacturer's instructions. For example, total RNA from cells in culture can be isolated using Qiagen RNeasy mini-columns. Other commercially available RNA isolation kits include MASTERPURE® Complete DNA and RNA Purification Kit (Epicentre, Madison, Wis.) and Paraffin Block RNA Isolation Kit (Ambion, Austin, Tex.). Total RNA from tissue samples can be isolated, for example, using RNA Stat-60 (Tel-Test, Friendswood, Tex.). RNA prepared from a tumor can be isolated, for example, by cesium chloride density gradient centrifugation. Additionally, large numbers of tissue samples can readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski (U.S. Pat. No. 4,843,155).

[0043] Isolated RNA can be used in hybridization or amplification assays that include, but are not limited to, PCR analyses and probe arrays. One method for the detection of RNA levels involves contacting the isolated RNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe can be, for example, a full-length cDNA, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 60, 100, 250, or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to an intrinsic gene of the present invention, or any derivative DNA or RNA. Hybridization of an mRNA with the probe indicates that the intrinsic gene in question is being expressed.

[0044] In one embodiment, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative embodiment, the probes are immobilized on a solid surface and the mRNA is contacted with the probes, for example, in an Agilent gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in detecting the level of expression of the intrinsic genes of the present invention.

[0045] An alternative method for determining the level of intrinsic gene expression product in a sample involves the process of nucleic acid amplification, for example, by RT-PCR (U.S. Pat. No. 4,683,202), ligase chain reaction (Barany, Proc. Natl. Acad. Sci. USA 88:189-93, 1991), self-sustained sequence replication (Guatelli et al., Proc. Natl. Acad. Sci. USA 87:1874-78, 1990), transcriptional amplification system (Kwoh et al., Proc. Natl. Acad. Sci. USA 86:1173-77, 1989), Q-Beta Replicase (Lizardi et al., Bio/Technology 6:1197, 1988), rolling circle replication (U.S. Pat. No. 5,854,033), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

[0046] In particular aspects of the invention, intrinsic gene expression is assessed by quantitative RT-PCR. Numerous different PCR or QPCR protocols are known in the art and exemplified herein below and can be directly applied or adapted for use using the presently-described compositions for the detection and/or quantification of the intrinsic genes listed in Table 1. Generally, in PCR, a target polynucleotide sequence is amplified by reaction with at least one oligonucleotide primer or pair of oligonucleotide primers. The primer(s) hybridize to a complementary region of the target nucleic acid and a DNA polymerase extends the primer(s) to amplify the target sequence. Under conditions sufficient to provide polymerase-based nucleic acid amplification products, a nucleic acid fragment of one size dominates the reaction products (the target polynucleotide sequence which is the amplification product). The amplification cycle is repeated to increase the concentration of the single target polynucleotide sequence. The reaction can be performed in any thermocycler commonly used for PCR. However, preferred are cyclers with real-time fluorescence measurement capabilities, for example, SMARTCYCLER® (Cepheid, Sunnyvale, Calif.), ABI PRISM 7700® (Applied Biosystems, Foster City, Calif.), ROTOR-GENE® (Corbett Research, Sydney, Australia), LIGHTCYCLER® (Roche Diagnostics Corp, Indianapolis, Ind.), ICYCLER® (Biorad Laboratories, Hercules, Calif.) and MX4000® (Stratagene, La Jolla, Calif.).

[0047] Quantitative PCR (QPCR) (also referred as real-time PCR) is preferred under some circumstances because it provides not only a quantitative measurement, but also reduced time and contamination. In some instances, the availability of full gene expression profiling techniques is limited due to requirements for fresh frozen tissue and specialized laboratory equipment, making the routine use of such technologies difficult in a clinical setting. However, QPCR gene measurement can be applied to standard formalin-fixed paraffin-embedded clinical tumor blocks, such as those used in archival tissue banks and routine surgical pathology specimens (Cronin et al. (2007) Clin Chem 53:1084-91)[Mullins 2007] [Paik 2004]. As used herein, "quantitative PCR (or "real time QPCR") refers to the direct monitoring of the progress of PCR amplification as it is occurring without the need for repeated sampling of the reaction products. In quantitative PCR, the reaction products may be monitored via a signaling mechanism (e.g., fluorescence) as they are generated and are tracked after the signal rises above a background level but before the reaction reaches a plateau. The number of cycles required to achieve a detectable or "threshold" level of fluorescence varies directly with the concentration of amplifiable targets at the beginning of the PCR process, enabling a measure of signal intensity to provide a measure of the amount of target nucleic acid in a sample in real time.

[0048] In another embodiment of the invention, microarrays are used for expression profiling. Microarrays are particularly well suited for this purpose because of the reproducibility between different experiments. DNA microarrays provide one method for the simultaneous measurement of the expression levels of large numbers of genes. Each array consists of a reproducible pattern of capture probes attached to a solid support. Labeled RNA or DNA is hybridized to complementary probes on the array and then detected by laser scanning. Hybridization intensities for each probe on the array are determined and converted to a quantitative value representing relative gene expression levels. See, for example, U.S. Pat. Nos. 6,040,138, 5,800,992 and 6,020,135, 6,033,860, and 6,344,316. High-density oligonucleotide arrays are particularly useful for determining the gene expression profile for a large number of RNAs in a sample.

[0049] Techniques for the synthesis of these arrays using mechanical synthesis methods are described in, for example, U.S. Pat. No. 5,384,261. Although a planar array surface is generally used, the array can be fabricated on a surface of virtually any shape or even a multiplicity of surfaces. Arrays can be nucleic acids (or peptides) on beads, gels, polymeric surfaces, fibers (such as fiber optics), glass, or any other appropriate substrate. See, for example, U.S. Pat. Nos. 5,770,358, 5,789,162, 5,708,153, 6,040,193 and 5,800,992. Arrays can be packaged in such a manner as to allow for diagnostics or other manipulation of an all-inclusive device. See, for example, U.S. Pat. Nos. 5,856,174 and 5,922,591.

[0050] In a specific embodiment of the microarray technique, PCR amplified inserts of cDNA clones are applied to a substrate in a dense array. The microarrayed genes, immobilized on the microchip, are suitable for hybridization under stringent conditions. Fluorescently labeled cDNA probes can be generated through incorporation of fluorescent nucleotides by reverse transcription of RNA extracted from tissues of interest. Labeled cDNA probes applied to the chip hybridize with specificity to each spot of DNA on the array. After stringent washing to remove non-specifically bound probes, the chip is scanned by confocal laser microscopy or by another detection method, such as a CCD camera. Quantitation of hybridization of each arrayed element allows for assessment of corresponding mRNA abundance.

[0051] With dual color fluorescence, separately labeled cDNA probes generated from two sources of RNA are hybridized pairwise to the array. The relative abundance of the transcripts from the two sources corresponding to each specified gene is thus determined simultaneously. The miniaturized scale of the hybridization affords a convenient and rapid evaluation of the expression pattern for large numbers of genes. Such methods have been shown to have the sensitivity required to detect rare transcripts, which are expressed at a few copies per cell, and to reproducibly detect at least approximately two-fold differences in the expression levels (Schena et al., Proc. Natl. Acad. Sci. USA 93:106-49, 1996). Microarray analysis can be performed by commercially available equipment, following manufacturer's protocols, such as by using the Affymetrix GenChip technology, or Agilent ink jet microarray technology. The development of microarray methods for large-scale analysis of gene expression makes it possible to search systematically for molecular markers of cancer classification and outcome prediction in a variety of tumor types.

Data Processing

[0052] It is often useful to pre-process gene expression data, for example, by addressing missing data, translation, scaling, normalization, weighting, etc. Multivariate projection methods, such as principal component analysis (PCA) and partial least squares analysis (PLS), are so-called scaling sensitive methods. By using prior knowledge and experience about the type of data studied, the quality of the data prior to multivariate modeling can be enhanced by scaling and/or weighting. Adequate scaling and/or weighting can reveal important and interesting variation hidden within the data, and therefore make subsequent multivariate modeling more efficient. Scaling and weighting may be used to place the data in the correct metric, based on knowledge and experience of the studied system, and therefore reveal patterns already inherently present in the data.

[0053] If possible, missing data, for example gaps in column values, should be avoided. However, if necessary, such missing data may replaced or "filled" with, for example, the mean value of a column ("mean fill"); a random value ("random fill"); or a value based on a principal component analysis ("principal component fill").

[0054] "Translation" of the descriptor coordinate axes can be useful. Examples of such translation include normalization and mean centering. "Normalization" may be used to remove sample-to-sample variation. For microarray data, the process of normalization aims to remove systematic errors by balancing the fluorescence intensities of the two labeling dyes. The dye bias can come from various sources including differences in dye labeling efficiencies, heat and light sensitivities, as well as scanner settings for scanning two channels. Some commonly used methods for calculating normalization factor include: (i) global normalization that uses all genes on the array; (ii) housekeeping genes normalization that uses constantly expressed housekeeping/invariant genes; and (iii) internal controls normalization that uses known amount of exogenous control genes added during hybridization (Quackenbush (2002) Nat. Genet. 32 (Suppl.), 496-501). In one embodiment, the intrinsic genes disclosed herein can be normalized to control housekeeping genes. For example, the housekeeping genes described in U.S. Patent Publication 2008/0032293, which is herein incorporated by reference in its entirety, can be used for normalization. Exemplary housekeeping genes include MRPL19, PSMC4, SF3A1, PUM1, ACTB, GAPD, GUSB, RPLP0, and TFRC. It will be understood by one of skill in the art that the methods disclosed herein are not bound by normalization to any particular housekeeping genes, and that any suitable housekeeping gene(s) known in the art can be used.

[0055] Many normalization approaches are possible, and they can often be applied at any of several points in the analysis. In one embodiment, microarray data is normalized using the LOWESS method, which is a global locally weighted scatterplot smoothing normalization function. In another embodiment, qPCR data is normalized to the geometric mean of set of multiple housekeeping genes.

[0056] "Mean centering" may also be used to simplify interpretation. Usually, for each descriptor, the average value of that descriptor for all samples is subtracted. In this way, the mean of a descriptor coincides with the origin, and all descriptors are "centered" at zero. In "unit variance scaling," data can be scaled to equal variance. Usually, the value of each descriptor is scaled by 1/StDev, where StDev is the standard deviation for that descriptor for all samples. "Pareto scaling" is, in some sense, intermediate between mean centering and unit variance scaling. In pareto scaling, the value of each descriptor is scaled by 1/sqrt(StDev), where StDev is the standard deviation for that descriptor for all samples. In this way, each descriptor has a variance numerically equal to its initial standard deviation. The pareto scaling may be performed, for example, on raw data or mean centered data.

[0057] "Logarithmic scaling" may be used to assist interpretation when data have a positive skew and/or when data spans a large range, e.g., several orders of magnitude. Usually, for each descriptor, the value is replaced by the logarithm of that value. In "equal range scaling," each descriptor is divided by the range of that descriptor for all samples. In this way, all descriptors have the same range, that is, 1. However, this method is sensitive to presence of outlier points. In "autoscaling," each data vector is mean centered and unit variance scaled. This technique is a very useful because each descriptor is then weighted equally, and large and small values are treated with equal emphasis. This can be important for genes expressed at very low, but still detectable, levels.

[0058] In one embodiment, data is collected for one or more test samples and classified using the PAM50 classification model described herein. When comparing data from multiple analyses (e.g., comparing expression profiles for one or more test samples to the centroids constructed from samples collected and analyzed in an independent study), it will be necessary to normalize data across these data sets. In one embodiment, Distance Weighted Discrimination (DWD) is used to combine these data sets together (Benito et al. (2004) Bioinformatics 20(1):105-114, incorporated by reference herein in its entirety). DWD is a multivariate analysis tool that is able to identify systematic biases present in separate data sets and then make a global adjustment to compensate for these biases; in essence, each separate data set is a multi-dimensional cloud of data points, and DWD takes two points clouds and shifts one such that it more optimally overlaps the other.

[0059] The methods described herein may be implemented and/or the results recorded using any device capable of implementing the methods and/or recording the results. Examples of devices that may be used include but are not limited to electronic computational devices, including computers of all types. When the methods described herein are implemented and/or recorded in a computer, the computer program that may be used to configure the computer to carry out the steps of the methods may be contained in any computer readable medium capable of containing the computer program. Examples of computer readable medium that may be used include but are not limited to diskettes, CD-ROMs, DVDs, ROM, RAM, and other memory and computer storage devices. The computer program that may be used to configure the computer to carry out the steps of the methods and/or record the results may also be provided over an electronic network, for example, over the internet, an intranet, or other network.

Calculation of Risk of Relapse

[0060] Provided herein are methods for predicting breast cancer outcome within the context of the intrinsic subtype and optionally other clinical variables. Outcome may refer to overall or disease-specific survival, event-free survival, or outcome in response to a particular treatment or therapy. In particular, the methods may be used to predict the likelihood of long-term, disease-free survival. "Predicting the likelihood of survival of a breast cancer patient" is intended to assess the risk that a patient will die as a result of the underlying breast cancer. "Long-term, disease-free survival" is intended to mean that the patient does not die from or suffer a recurrence of the underlying breast cancer within a period of at least five years, or at least ten or more years, following initial diagnosis or treatment.

[0061] In one embodiment, outcome is predicted based on classification of a subject according to subtype. This classification is based on expression profiling using the list of intrinsic genes listed in Table 1. As discussed in Example 1, tumor subtype according to the PAM50 model was more indicative of response to chemotherapy than standard clinical marker classification. Tumors classified as HER2+ using clinical markers but not HER2-enriched using the PAM50 model had a lower pathological complete response (pCR) to a regimen of paclitaxel, 5-fluorouracil, adriamycin, and cyclophosphamide (T/FAC) than tumors classified as HER2+ clinically and belonging to the HER2-enriched expression subtype. Similarly, Basal-like tumors that were not clinically scored as triple-negative (ER−, PgR− and HER2−) had a higher pCR compared to triple-negative tumors that were not Basal-like by PAM50. Thus, the PAM50 model can be used to more accurately predict response to chemotherapy than standard clinical markers.

[0062] In addition to providing a subtype assignment, the PAM50 bioinformatics model provides a measurement of the similarity of a test sample to all four subtypes which is translated into a Risk Of Relapse (ROR) score that can be used in any patient population regardless of disease status and treatment options. The intrinsic subtypes and ROR also have value in the prediction of pathological complete response in women treated with, for example, neoadjuvant taxane and anthracycline chemotherapy [Rouzier 2005]. Thus, in various embodiments of the present invention, a risk of relapse (ROR) model is used to predict outcome. Using these risk models, subjects can be stratified into low, medium, and high risk of relapse groups. Calculation of ROR can provide prognostic information to guide treatment decisions and/or monitor response to therapy.

[0063] In some embodiments described herein, the prognostic performance of the PAM50-defined intrinsic subtypes and/or other clinical parameters is assessed utilizing a Cox Proportional Hazards Model Analysis, which is a regression method for survival data that provides an estimate of the hazard ratio and its confidence interval. The Cox model is a well-recognized statistical technique for exploring the relationship between the survival of a patient and particular variables. This statistical method permits estimation of the hazard (i.e., risk) of individuals given their prognostic variables (e.g., intrinsic gene expression profile with or without additional clinical factors, as described herein). The "hazard ratio" is the risk of death at any given time point for patients displaying particular prognostic variables. See generally Spruance et al., Antimicrob. Agents & Chemo. 48:2787-92, 2004.

[0064] The PAM50 classification model described herein can be trained for risk of relapse using subtype distances (or correlations) alone, or using subtype distances with clinical variables as discussed supra. In one embodiment, the risk score for a test sample is calculated using intrinsic subtype distances alone using the following equation: ROR=0.05*Basal+0.11*Her2+-0.25*LumA+0.07*LumB+−0.11*Normal, where the variables "Basal," "Her2," "LumA," "LumB," and "Normal" are the distances to the centroid for each respective classifier when the expression profile from a test sample is compared to centroids constructed using the gene expression data deposited with the Gene Expression Omnibus (GEO) as accession number GSE2845. It is also possible that other data sets could be used to derive similar Cox Model coefficients. When using the intrinsic gene list set forth in Table 1 to develop a prediction model from a sample set other than the samples used to derive the dataset deposited as GSE2845, the methods described in Example 1 or Example 3 can be used to construct a formula for calculating the risk of relapse from this alternate sample set.

[0065] Risk score can also be calculated using a combination of breast cancer subtype and the clinical variables tumor size (T) and lymph nodes status (N) using the following equation: ROR (full)=0.05*Basal+0.1*Her2+−0.19*LumA+0.05*LumB+−0.09*Normal+0.16*T+0.08*−N, again when comparing test expression profiles to centroids constructed using the gene expression data deposited with GEO as accession number GSE2845.

[0066] In yet another embodiment, risk score for a test sample is calculated using intrinsic subtype distances alone using the following equation: ROR-S=0.05*Basal+0.12*Her2+−0.34*LumA+0.0.23*LumB,

[0067] where the variables "Basal," "Her2," "LumA," and "LumB" are as described supra and the test expression profiles are compared to centroids constructed using the gene expression data deposited with GEO as accession number GSE2845.

[0068] In yet another embodiment, risk score can also be calculated using a combination of breast cancer subtype and the clinical variable tumor size (T) using the following equation (where the variables are as described supra): ROR-C=0.05*Basal+0.11*Her2+−0.23*LumA+0.09*LumB+0.17*T.

Prediction of Response to Therapy

[0069] Breast cancer is managed by several alternative strategies that may include, for example, surgery, radiation therapy, hormone therapy, chemotherapy, or some combination thereof. As is known in the art, treatment decisions for individual breast cancer patients can be based on endocrine responsiveness of the tumor, menopausal status of the patient, the location and number of patient lymph nodes involved, estrogen and progesterone receptor status of the tumor, size of the primary tumor, patient age, and stage of the disease at diagnosis. Analysis of a variety of clinical factors and clinical trials has led to the development of recommendations and treatment guidelines for early-stage breast cancer by the International Consensus Panel of the St. Gallen Conference (2005). See, Goldhirsch et al., Annals Oncol. 16:1569-83, 2005. The guidelines recommend that patients be offered chemotherapy for endocrine non-responsive disease; endocrine therapy as the primary therapy for endocrine responsive disease, adding chemotherapy for some intermediate- and all high-risk groups in this category;

and both chemotherapy and endocrine therapy for all patients in the uncertain endocrine response category except those in the low-risk group.

[0070] Stratification of patients according to risk of relapse using the PAM50 model and risk score disclosed herein provides an additional or alternative treatment decision-making factor. The methods comprise evaluating risk of relapse using the PAM50 classification model optionally in combination with one or more clinical variables, such as node status, tumor size, and ER status. The risk score can be used to guide treatment decisions. For example, a subject having a low risk score may not benefit from certain types of therapy, whereas a subject having a high risk score may be indicated for a more aggressive therapy.

[0071] The methods of the invention find particular use in choosing appropriate treatment for early-stage breast cancer patients. The majority of breast cancer patients diagnosed at an early-stage of the disease enjoy long-term survival following surgery and/or radiation therapy without further adjuvant therapy. However, a significant percentage (approximately 20%) of these patients will suffer disease recurrence or death, leading to clinical recommendations that some or all early-stage breast cancer patients should receive adjuvant therapy. The methods of the present invention find use in identifying this high-risk, poor prognosis population of early-stage breast cancer patients and thereby determining which patients would benefit from continued and/or more aggressive therapy and close monitoring following treatment. For example, early-stage breast cancer patients assessed as having a high risk score by the methods disclosed herein may be selected for more aggressive adjuvant therapy, such as chemotherapy, following surgery and/or radiation treatment. In particular embodiments, the methods of the present invention may be used in conjunction with the treatment guidelines established by the St. Gallen Conference to permit physicians to make more informed breast cancer treatment decisions.

[0072] In various embodiments, the PAM50 classification model provides information about breast cancer subtypes that cannot be obtained using standard clinical assays such as immunohistochemistry or other histological analyses. For example, subjects scored as estrogen receptor (ER)-positive and/or progesterone-receptor (PR)-positive would be indicated under conventional guidelines for endocrine therapy. As discussed in Example 2, the model disclosed herein is capable of identifying a subset of these ER+/PgR+ cases that are classified as Basal-like, which may indicate the need for more aggressive therapy that would not have been indicated based on ER or PgR status alone.

[0073] Thus, the methods disclosed herein also find use in predicting the response of a breast cancer patient to a selected treatment. "Predicting the response of a breast cancer patient to a selected treatment" is intended to mean assessing the likelihood that a patient will experience a positive or negative outcome with a particular treatment. As used herein, "indicative of a positive treatment outcome" refers to an increased likelihood that the patient will experience beneficial results from the selected treatment (e.g., complete or partial remission, reduced tumor size, etc.). "Indicative of a negative treatment outcome" is intended to mean an increased likelihood that the patient will not benefit from the selected treatment with respect to the progression of the underlying breast cancer.

[0074] In some embodiments, the relevant time for assessing prognosis or disease-free survival time begins with the surgical removal of the tumor or suppression, mitigation, or inhibition of tumor growth. In another embodiment, the PAM50-based risk score is calculated based on a sample obtained after initiation of neoadjuvant therapy such as endocrine therapy. The sample may be taken at any time following initiation of therapy, but is preferably obtained after about one month so that neoadjuvant therapy can be switched to chemotherapy in unresponsive patients. It has been shown that a subset of tumors indicated for endocrine treatment before surgery is non-responsive to this therapy. The model provided herein can be used to identify aggressive tumors that are likely to be refractory to endocrine therapy, even when tumors are positive for estrogen and/or progesterone receptors. In this embodiment, a proliferation-weighted PAM50 risk score is obtained according the following equation: RSp=(−0.0129*Basal)+(0.106*Her2)+(−0.112*LumA)+(0.039*LumB)+(−0.069*Norma-1)+(0.272*Prolif), where the proliferation score ("prolif") is assigned as the mean measurement of the following genes (after normalization): CCNB1, UBE2C, BIRCS, KNTC2, CDC20, PTTG1, RRM2, MKI67, TYMS, CEP55, and CDCA1. All other variables are the same as the RS equations described infra. As discussed in Example 2, assessment of risk score after initiation of therapy is more predictive of outcome to treatment, at least in a population of ER+ patients undergoing neoadjuvant endocrine therapy.

Kits

[0075] The present invention also provides kits useful for classifying breast cancer intrinsic subtypes and/or providing prognostic information. These kits comprise a set of capture probes and/or primers specific for the intrinsic genes listed in Table 1, as well as reagents sufficient to facilitate detection and/or quantitation of the intrinsic gene expression product. The kit may further comprise a computer readable medium.

[0076] In one embodiment of the present invention, the capture probes are immobilized on an array. By "array" is intended a solid support or a substrate with peptide or nucleic acid probes attached to the support or substrate. Arrays typically comprise a plurality of different capture probes that are coupled to a surface of a substrate in different, known locations. The arrays of the invention comprise a substrate having a plurality of capture probes that can specifically bind an intrinsic gene expression product. The number of capture probes on the substrate varies with the purpose for which the array is intended. The arrays may be low-density arrays or high-density arrays and may contain 4 or more, 8 or more, 12 or more, 16 or more, 32 or more addresses, but will minimally comprise capture probes for the 50 intrinsic genes listed in Table 1.

[0077] Techniques for the synthesis of these arrays using mechanical synthesis methods are described in, e.g., U.S. Pat. No. 5,384,261, incorporated herein by reference in its entirety for all purposes. The array may be fabricated on a surface of virtually any shape or even a multiplicity of surfaces. Arrays may be probes (e.g., nucleic-acid binding probes) on beads, gels, polymeric surfaces, fibers such as fiber optics, glass or any other appropriate substrate, see U.S. Pat. Nos. 5,770,358, 5,789,162, 5,708,153, 6,040,193 and 5,800,992, each of which is hereby incorporated in its entirety for all purposes. Arrays may be packaged in such a manner as to allow for diagnostics or other manipulation on the device. See, for example, U.S. Pat. Nos. 5,856,174 and 5,922,591 herein incorporated by reference.

[0078] In another embodiment, the kit comprises a set of oligonucleotide primers sufficient for the detection and/or quantitation of each of the intrinsic genes listed in Table 1. The oligonucleotide primers may be provided in a lyophilized or reconstituted form, or may be provided as a set of nucleotide sequences. In one embodiment, the primers are provided in a microplate format, where each primer set occupies a well (or multiple wells, as in the case of replicates) in the microplate. The microplate may further comprise primers sufficient for the detection of one or more housekeeping genes as discussed infra. The kit may further comprise reagents and instructions sufficient for the amplification of expression products from the genes listed in Table 1.

[0079] In order to facilitate ready access, e.g., for comparison, review, recovery, and/or modification, the molecular signatures/expression profiles are typically recorded in a database. Most typically, the database is a relational database accessible by a computational device, although other formats, e.g., manually accessible indexed files of expression profiles as photographs, analogue or digital imaging readouts, spreadsheets, etc. can be used. Regardless of whether the expression patterns initially recorded are analog or digital in nature, the expression patterns, expression profiles (collective expression patterns), and molecular signatures (correlated expression patterns) are stored digitally and accessed via a database. Typically, the database is compiled and maintained at a central facility, with access being available locally and/or remotely.

[0080] The article “a” and “an” are used herein to refer to one or more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one or more element.

[0081] Throughout the specification the word “comprising,” or variations such as “comprises” or “comprising,” will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

[0082] The following examples are offered by way of illustration and not by way of limitation:

EXPERIMENTAL

Example 1

Methods

Samples and Clinical Data:

[0083] Patient cohorts for traiing and test sets consisted of samples with data already in the public domain (Loi et al. (2007) J. Clin. Oncol. 25:1239-1246; va de Vijver et al. (2002) N Engl J Med 247:1999-2009; Wang et al (2005) Lancet 365:671-679; Ishvina et al. (2006) Cancer Res 66:10292-10301; and Hess et al (2006) J Clin Oncol 24:4236-4244, each of which is incorporated by reference in its entirety) and fresh frozen and formalin-fixed paraffin-embedded (FFPE) tissues collected under institutional review board-approved protocols at the respective institutions.

[0084] A training set of 189 breast tumor samples and 29 normal samples was procured as fresh frozen and FFPE tissues under approved IRB protocols at the University of North Carolina at Chapel Hill, The University of Utah, Thomas Jefferson University, and Washington University. The training set, which was gene expression profiled by microarray and qRT-PCR, had a median follow-up of 49 months and represents heterogeneously treated patients in accordance with the standard of care dictated by their stage, ER and HER2 status. A test set of 279 breast cancers with long-term, disease-specific survival was gene expression profiled from FFPE by qRT-PCR. The clinical data for the training and test set assayed by qRT-PCR are provided in Tables 2 and 3.

Nucleic Acid Extraction:

[0085] Total RNA was purified from fresh frozen samples for microarray using the Qiagen RNeasy Midi Kit according to the manufacturer’s protocol (Qiagen, Valencia Calif.). The integrity of the RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif.). The High Pure RNA Paraffin Kit (Roche Applied Science, Indianapolis, Ind.) was used to extract RNA from FFPE tissues (2×10 micron or 1.5 mm punches) for qRT-PCR. Contaminating DNA was removed using Turbo DNase (Ambion, Austin, Tex.). The yield of total RNA was assessed using the Nanoprop ND-1000 Spectrophotometer (Nanoprop Technologies, Inc., Rockland, Del.).

Reverse Transcription and Real-Time Quantitative PCR:

[0086] First-strand cDNA was synthesized from 1.2 μg total RNA using Superscript III reverse transcriptase (1st Strand Kit; Invitrogen, Carlsbad, Calif.) and a mixture of random hexamers and gene specific primers. The reaction was held at 55° C. for 60 minutes and then 70° C. for 15 minutes. The cDNA was washed on a QIAquick PCR purification column (Qiagen Inc., Valencia, Calif.) and stored at −80° C. in 25 mM Tris, 1 mM EDTA until further use. Each 54, PCR reaction included 1.25 ng (0.625 ng/μL) cDNA from samples of interest or 10 ng (5 ng/μL) for reference, 2 pmol of both upstream and downstream primers, and 2× LightCycler 480 SYBR Green I Master Mix (Roche Applied Science, Indianapolis, Ind.). Each run contained a single gene profiled in duplicate for test samples, reference sample, and negative control. The reference sample cDNA was comprised of an equal contribution of Human Reference Total RNA (Stratagene, La Jolla, Calif.) and the breast cell lines MCF7, ME16C, and SKBR3. PCR amplification was performed with the LightCycler 480 (Roche Applied Science, Indianapolis, Ind.) using an initial denaturation step (95° C., 8 minutes) followed by 45 cycles of denaturation (95° C., 4 seconds), annealing (56° C., 6 seconds with 2.5° C./s transition), and extension (72° C., 6 seconds with 2° C./sec transition). Fluorescence (530 nm) from the dsDNA dye SYBR Green I was acquired each cycle after the extension step. The specificity of the PCR was determined by post-amplification melting curve analysis—samples were cooled to 65° C. and slowly heated at 2° C./s to 99° C. while continuously monitoring fluorescence (10 acquisitions/1° C.). The relative copy number for each gene was determined from a within run calibrator set at 10 ng and using a PCR efficiency of 1.9. Each of the PAM50 classifier genes was normalized to the geometric mean of 5 housekeepers.

Microarray:

[0087] Total RNA isolation, labeling and hybridizations on Agilent human 1Av2 microarrays or custom designed Agilent human 22 k arrays were performed using the protocol described in Hu et al (6). All microarray data have been deposited into the GEO under the accession number of GSE10886. Sources for all microarray training and test data sets are given in Table 4.

Pre-Processing of Microarray Data:

[0088] Microarray data for the training set (189 samples) were extracted from the University of North Carolina (UNC) microarray database. Raw signal intensities from both channels were lowess normalized by chip and probes were excluded from data analysis if they did not have signal intensity of at least 30 in both channels for at least 70% of the experiments. The normalized data for this set have been placed on GEO (GSE10886). The training set was median-centered and gene symbols were assigned using the manufacturer provided annotation. Duplicate gene symbols were collapsed by averaging within each sample.

[0089] Normalized data for all test sets were downloaded from GEO (GSE2845, GSE6532, GSE4922, GSE2034, GSE10886) or the publicly-available data found at the world wide web (www) at bioinformatics.mdanderson [dot] org/pubdata (see Table 5). All intensity measures (ratios for the NKI data) were log-transformed. Prior to nearest centroid calculation, the Hess et al. (see the world wide web (www) at bioinformatics.mdanderson [dot] org/pubdata), van de Vijver et al. (GSE2845), and Wang et al. (GSE2034) datasets were median centered to minimize platform effects. Adjustment in this way assumes a relatively similar sampling of the population as the training set. The Loi et al. (GSE6532) and Ivshina et al. (GSE4922) datasets were heavily enriched for ER+ samples relative to the training set, thus the underlying assumption may be violated for these sets. In these two instances the genes in the training set were centered to the median of the ER+ samples (as opposed to the median across all samples). As with the training set, gene symbols were assigned using the manufacturer provided annotation, and duplicate gene symbols were collapsed by averaging within each sample.

Identification of Prototypical Intrinsic Subtype Samples and Genes:

[0090] An expanded "intrinsic" gene set, comprised primarily of genes found in 4 previous studies (1, 6, 9, 11), was initially used to identify prototypical tumor samples. The Normal-like class was represented using true "normals" from reduction mammoplasty or grossly uninvolved tissue. 189 breast tumors across 1906 "intrinsic" genes were analyzed by hierarchical clustering (median centered by feature/gene, Pearson correlation, average linkage) (12) and the sample dendrogram was analyzed using "SigClust" (13). The SigClust algorithm statistically identifies significant/unique groups by testing the null hypothesis that a group of samples is from a single cluster, where a cluster is characterized as a multivariate normal distribution. SigClust was run at each node of the dendrogram beginning at the root and stopping when the test was no longer significant ($p>0.001$).

Gene Set Reduction Using Prototype Samples and qRT-PCR:

[0091] 122 breast cancers from 189 individuals profiled by qRT-PCR and microarray had prototypical profiles as determined by SigClust (Table 2). A minimized gene set was derived from these prototypical samples using the qRT-PCR data for 161 genes that passed FFPE performance criteria established in Mullins et al (14). Several minimization methods were employed including top "N" t-test statistics for each group (15), top cluster index scores (16), and the remaining genes after 'shrinkage' of modified t-test statistics (17). Cross-validation (random 10% left out in each of 50 cycles) was used to assess the robustness of the minimized gene sets. The "N" t-test method was selected due to having the lowest CV error.

Sample Subtype Prediction:

[0092] Minimized gene sets were compared for reproducibility of classification across 3 centroid-based prediction methods: Prediction Analysis of Microarray (PAM) (17), a simple nearest centroid (6), and Classification of Nearest Centroid (ClaNC) (18). Subtype prediction was done by calculating the Spearman's rank correlation of each test case to five centroids (LumA, LumB, HER2-enriched, Basal-like, and Normal-like) and class was assigned based upon the nearest centroid. Centroids were constructed as described for the PAM algorithm (17) using the data provided in GSE10886; however, no "shrinkage" was used and the Spearman's rank correlation was used for the distance measure. This method was selected as the classifier because of its reproducibility of subtype predictions from large and minimized gene sets. The final 50-gene classifier (henceforth called PAM50) was used to make subtype predictions onto 6 microarray datasets and 1 qRT-PCR dataset (Table 4). The Hess et al dataset (19) does not have outcome data and is evaluated based on clinical markers, subtypes, and neo-adjuvant response.

Prognosis Using Clinical and Molecular Subtype Data:

[0093] The prognostic significance of the intrinsic subtype classification was assessed along with standard clinical variables (tumor size (T), node status (N), and ER status) using univariate and multivariate analyses with time to relapse (i.e., any event) as the endpoint. Likelihood ratio tests were performed to compare models of available clinical data, subtype data, and combined clinical and molecular variables. Categorical survival analyses were performed using a log rank test and visualized with Kaplan-Meier plots.

Developing Risk Models with Clinical and Molecular Data:

[0094] Models were trained for risk of relapse (ROR) predictions using subtype alone, and subtype with clinical information. In both cases, a multivariate Cox model using Ridge regression was fit to the untreated subset of the NKI295 cohort (20). A risk score was assigned to each test case using correlation to the subtype alone (ROR; model 1) or using a full model with subtype correlation and two clinical variables (ROR (full); model 2):

[0095] (1) ROR=0.05*Basal+0.11*Her2+−0.25*LumA+0.07*LumB+−0.11*Normal

[0096] (2) ROR(full)=0.05*Basal+0.1*Her2+−0.19*LumA+0.05*LumB+−0.09*Normal+0.16*T+0-0.08*N

[0097] The sum of the coefficients from the Cox model is the "risk of relapse" score for each patient. In order to classify samples into specific risk groups, thresholds were chosen from the NKI training set that required no LumA sample to be in the high risk group and no Basal-like sample to be in the low risk group. Thresholds were determined from the training set and remained unchanged when evaluating test cases. Predictions for the subtype only and combined models were compared using the C Index (see the world wide web (www) at lib.stat.cmu [dot] edu/S/Harrell/Design.html). SiZer analysis was performed to characterize the relationship between the ROR score and relapse free survival (21). The 95% confidence intervals for the ROR score are local versions of binomial confidence intervals, with the local sample size computed from a Gaussian kernel density estimator, based on the Sheather-Jones choice of window width (22).

Results

Creating a New Subtype Model Based Upon Prototypical Samples and Genes:

[0098] There have been numerous studies that have analyzed interactions between breast cancer intrinsic subtypes and prognosis (1, 6, 9), genetic alterations (23), and drug response (24). The purpose of the methods described here was to standardize and validate a classification for the intrinsic subtypes for clinical and research purposes. "SigClust" objectively identified five intrinsic breast subtypes from clustered microarray data. These prototypes were then used to derive a minimal 50-gene set (PAM50). Finally, the best classification method was selected and used with the PAM50 to predict subtypes on multiple test sets from microarray and qRT-PCR data. Of the 5 microarray studies with outcome data (Table 4), the UNC cohort had significantly worse outcomes than the others. Subtype predictions onto a combined microarray test set showed prognostic significance across all patients, in patients given endocrine treatment alone, and in node negative patients receiving no systemic adjuvant therapy (FIGS. **1**A to **1**C).

[0099] Molecular and clinical predictors of survival were assessed in univariate and multivariate analyses on 1451 patients (Table 5). In univariate analysis, the LumA, LumB, and HER2-enriched subtypes were all found to be significant, as were the clinical variables ER, T, and N. The LumA and HER2-enriched subtypes and the clinical variables were also significant in multivariate analyses, suggesting that the most comprehensive model should include subtype and clinical information. Testing this hypothesis revealed that the combined model accounts for significantly more variation in survival than either the subtype or clinical variables alone ($p<0.0001$ for both tests).

Distribution of Biological Subtypes Across ER Positive and ER-Negative Tumors:

[0100] Of all ER-positive tumors in the combined microarray test set, 73% were Luminal (A and B), 10% were HER2-enriched, and 5% were Basal-like (Table 6). Conversely when ER-negative tumors were considered, approximately 13% were Luminal (A and B), 31% were HER2-enriched and 48% were Basal-like. Tumors identified as the Normal-like subtype were divided almost equally between ER-positive (11%) and ER-negative (8%) tumors. Therefore, while subtype representation markedly changed in distribution depending on ER-status, all subtypes were represented in both ER-positive and ER-negative categories. Outcome plots for the subtypes in ER-positive cases alone were significant for relapse free survival and followed the same trends as seen when considering all invasive breast disease.

Subtypes and Response to Neoadjuvant T/FAC Treatment:

[0101] The Hess et al. study that performed microarray on tumors from patients given a regimen of paclitaxel, 5-fluorouracil, adriamycin, and cyclophosphamide (T/FAC) (19) allowed investigation of the relationship between the PAM50 subtypes, clinical markers, and how each relates to pathological complete response (pCR). For HER2 status, 64% of tumors that were HER2-positive by clinical assay (FISH+ and/or IHC 3+, referred to as HER2+ clin) were classified into the HER2-enriched expression subtype, with the rest of the HER2+ clin mostly associated with the Luminal subtypes. Tumors that were HER2+ clin but not of the HER2-enriched expression subtype had a low pCR rate (16%) versus those that were HER2+ clin and HER2-enriched expression subtype (52%).

[0102] Another relevant clinical distinction is the classification of "triple-negative" tumors (ER−, PgR− and HER2−), of which 65% were called Basal-like by the PAM50, with the remainder being called HER2-enriched (15%), LumA (4%), LumB (4%), and Normal-like (12%). The PAM50 classification of Basal-like appears superior to the clinical triple-negative with respect to pCR rate in that Basal-like tumors that were not scored as triple-negative had a 50% pCR compared to triple-negative tumors that were not Basal-like by PAM50 (22% pCR, Table 7).

Risk Prediction Based on Biological Subtype:

[0103] A supervised risk classifier was developed to predict outcomes within the context of the intrinsic subtypes and clinical variables. An untreated cohort was selected from the NKI microarray dataset to train the risk of relapse (ROR) model and select cut-offs. Two Cox models (one based upon subtype alone and another based upon subtype, tumor size, and node status) were validated using the combined microarray test set. Excluding clinical variables, the subtype only model performed well at stratifying patients into low, medium, and high risk of relapse groups (c-index=0.65 [0.61-0.69]); however, the full model (subtype, tumor size, node status) performed better (c-index=0.70 [0.66-0.74]), and, in practice, stage is a parameter that needs to be accounted for (FIGS. **2**A to **2**D). FIGS. **3**A and **3**B show the probability of relapse-free survival at 5 years plotted as a continuous linear scale using the full model.

[0104] The PAM50 classifier, assayed by qRT-PCR, was applied to a heterogeneously treated cohort archived between 1976 and 1995. The subtype classifications followed the same survival trends as seen in the microarray data and the ROR score was significant for long-term relapse predictions. This old age sample set was also scored for standard clinical markers (ER and HER2) by immunohistochemistry (IHC) and compared to the gene expression-based test. Analysis of ESR1 and ERBB2 by gene expression showed high sensitivity and specificity as compared to the IHC assay.

Discussion

[0105] The PAM50 classifier was developed using a statistically derived gene and sample set and was validated across multiple cohorts and platforms with the intent of delivering a clinical diagnostic test for the intrinsic subtypes of breast cancer. The large and diverse test sets allowed evaluation of the performance of the assay at a population level and in relation to standard molecular markers. An important finding from these analyses is that all of the intrinsic subtypes are present within both clinically defined ER-positive and ER-negative tumor subsets, with the subtype designations in the ER-positive patients showing prognostic significance. Thus, the molecular subtypes are not simply another method of classification based upon ER status.

[0106] There were also other important findings concerning individual subtypes. For example, some of the tumors classified into the HER2-enriched expression subtype were not HER2+ clin, suggesting the presence of an ER-negative non-Basal subtype that is not driven by HER2 gene amplification. It was also found that about 10% of breast cancers were classified as Normal-like and can be either ER-positive or ER-negative and have an intermediate prognosis. Since these tumors were predicted by training on normal breast tissue, the Normal-like class may be an artifact of having a high percentage of normal “contamination” in the tumor specimen. Other possibilities are that these are slow growing Basal-like tumors that lack high expression of the proliferation genes, or are a potential new subtype that has been referred to as claudin-low tumors (25). Detailed histological, immunohistochemical, and additional gene expression analyses of these cases are needed to resolve these issues.

[0107] Discrepancies between subtype and standard molecular markers have important therapeutic implications. For instance, a patient with a Basal-like subtype tumor that was scored ER or PgR-positive would likely be treated by endocrine therapy and would not be eligible for protocols that aim to develop Basal-like specific therapies (e.g., platinum containing regimens). These analyses of the Hess et al. dataset (19) showed that no patient with the LumA subtype had a pCR when administered an aggressive neoadjuvant regimen whereas the pCR rate of the Basal-like tumors was 59%. Furthermore, there has been debate about whether the triple-negative (ER−, PR−, HER2−) phenotype is the same as the Basal-like expression subtype26. A recent tissue microarray study of 3744 tumors confirmed the poor prognosis of triple-negative cases, but also revealed that tumors lacking all markers did not behave the same as those that were positive for one or two Basal-like markers (i.e., CK5/6 or HER1) (27). In agreement with the idea that the Basal-like diagnosis should be made independent of clinical ER and PgR status, a higher therapeutic response to T/FAC was found in those subjects identified as Basal-like but non-triple negative (50%) versus those identified as triple-negative but not Basal-like (22%). This suggests that the Basal-like subtype designation may ultimately prove superior to the triple-negative definition in identifying tumors with a high degree of chemotherapy sensitivity.

[0108] Providing an absolute subtype classification is somewhat artificial as tumors do not exist as discrete biological entities. Classification of tumors into low-medium-high risk groups based upon distance to each subtype centroid (i.e., the ROR model) was an attempt to deal with this issue and yielded significant survival segregation. This was true when combining all test cases, or after stratification into cohorts given endocrine therapy only, or no systemic adjuvant treatment. One of the major benefits of the ROR predictor is the identification of LumA patients that are at a very low risk of relapse, and for whom the benefit from adjuvant chemotherapy is unlikely. In this context the ROR predictor based on subtypes provides similar information as the OncotypeDx Recurrence Score for ER-positive, node negative patients (4, 5). However the PAM50 based assay provides a risk of relapse score for all patients, including those with ER-negative disease.

[0109] In summary, this subtype predictor and ROR classifier effectively identifies molecular features in breast tumors that are important for prognosis and treatment. The qRT-PCR assay can be performed using archived breast tissues, which will be useful for retrospective studies and prospective clinical trials.

TABLE 2

Clinical and Subtype Data for Prototype Samples from Microarray/qRT-PCR Training Sets

Patient ID	GEO accession	Subtype Assignment (SigClust)	qPCR_name	Dx Age	Ethnicity	pT*	pN^	M	Grade%	Overall Survival	Vital Status	ER (IHC)**	PR (IHC)**	HER2 (status)^^
1	GSM275694	Basal-like	BR000161BPE_UU	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	GSM140985	Basal-like	BR000572BPE_UU	45	AA	3	0	0	3	NA	NA	0	0	NA
7	GSM140999	Basal-like	BR010235BPE_UU	36	AA	1	0	0	3	49	0	0	0	0
10	GSM275782	Basal-like	BR010532BPE_UU	47	AA	3	1	0	3	14	1	0	0	0
11	GSM80221	Basal-like	BR020018BPE_UU	55	C	2	0	0	3	31	0	0	0	0
12	GSM141096	HER2-enriched	BR020155BPE_UU	38	NA	3	1	0	3	42	0	0	0	1
13	GSM141099	HER2-enriched	BR020306BPE_UU	42	C	4	2	1	3	22	1	1	1	1
14	GSM141102	LumB	BR020439BPE_UU	53	C	4	1	1	3	16	1	1	0	0
15	GSM275783	LumA	BR020464BPE_UU	44	C	2	0	0	1	2	0	1	1	0
16	GSM141105	Basal-like	BR020578BPE_UU	76	AA	4	0	0	3	5	1	0	0	1
18	GSM141110	Basal-like	BR030459BPE_UU	30	C	3	0	0	3	37	0	0	0	0
19	GSM275771	LumA	BR030584BPE_UU	54	C	1	1	NA	NA	NA	0	1	1	0
21	GSM141114	LumB	BR040114BPE_UU	56	C	2	0	0	2	16	0	1	1	0
22	GSM141117	LumB	BR040182BPE_UU	88	C	2	1	0	3	16	0	1	1	1
23	GSM141121	HER2-enriched	BR040269BPE_UU	46	C	2	0	0	3	17	0	0	0	1

TABLE 2-continued

Clinical and Subtype Data for Prototype Samples from Microarray/qRT-PCR Training Sets

Patient ID	GEO accession	Subtype Assignment (SigClust)	qPCR_name	Dx Age	Ethnicity	pT*	pN^	M	Grade%	Overall Survival	Vital Status	ER (IHC)**	PR (IHC)**	HER2 (status)^^
28	GSM34523	Basal-like	PB00205PE_UU	39	C	NA	NA	1	3	5	1	0	0	0
29	GSM52895	LumA	PB00284PE_UU	34	C	1	0	0	1	54	0	1	1	0
30	GSM34565	Basal-like	PB00297PE_UU	55	AA	2	0	0	3	55	0	0	0	0
31	GSM34481	HER2-enriched	PB00311PE_UU	47	C	2	1	0	3	50	0	1	1	0
32	GSM34497	HER2-enriched	PB00314PE_UU	50	C	3	1	0	3	52	0	0	0	1
33	GSM34527	Basal-like	PB00334PE_UU	50	AA	1	0	0	3	54	0	0	0	0
35	GSM34544	HER2-enriched	PB00376PE_UU	50	AA	2	0	0	3	49	0	0	0	0
37	GSM34549	LumA	PB00441PE_UU	83	C	1	0	0	2	14	0	1	1	0
38	GSM34528	HER2-enriched	PB00455PE_UU	52	AA	3	1	0	2	46	0	0	0	1
39	GSM52884	LumA	PB00479PE_UU	50	NA	2	0	0	NA	NA	0	1	1	0
41	GSM50157	Basal-like	UB00028PE_UU	46	C	1	0	0	3	59	0	0	0	0
42	GSM34437	Basal-like	UB00029PE_UU	59	C	2	0	0	3	59	0	0	0	0
43	GSM34431	HER2-enriched	UB00037PE_UU	42	C	1	1	0	3	58	0	0	1	0
44	GSM34548	LumA	UB00038PE_UU	50	C	1	0	0	2	57	0	1	1	0
47	GSM34428	LumA	UB00044PE_UU	49	C	2	1	0	2	59	0	1	1	0
50	GSM34557	LumA	UB00056PE_UU	63	C	1	1	0	2	56	0	1	1	0
53	GSM34532	HER2-enriched	UB00060PE_UU	72	C	3	3	0	3	49	0	0	0	1
56	GSM34450	Basal-like	UB00067PE_UU	80	C	1	1	0	3	38	1	0	0	0
57	GSM34451	LumA	UB00069PE_UU	40	C	1	0	0	2	7	0	NA	NA	0
58	GSM34452	Basal-like	UB00071PE_UU	60	C	1	0	0	3	50	0	0	0	0
60	GSM141079	LumA	UB00081LPE_UU	65	C	NA	1	0	2	44	0	1	1	0
61	GSM141081	LumA	UB00082PE_UU	43	C	1	1	0	1	40	0	1	1	0
63	GSM141084	LumB	UB00088PE_UU	69	C	2	2	0	2	38	1	1	1	1
64	GSM141085	LumA	UB00091PE_UU	77	C	1	0	0	2	36	0	1	1	0
65	GSM141088	LumA	UB00099PE_UU	50	C	3	1	0	2	35	0	1	1	0
66	GSM141070	Basal-like	UB00100PE_UU	49	C	1	0	0	3	34	0	NA	NA	0
67	GSM141071	Basal-like	UB00110PE_UU	76	C	2	0	0	3	31	0	0	0	0
68	GSM141072	Basal-like	UB00116PE_UU	67	C	2	0	NA	3	34	0	0	0	1
69	GSM141073	HER2-enriched	UB00117PE_UU	72	other	NA	0	0	3	31	0	0	0	1
72	GSM275802	Basal-like	WU00328-16563PE_UU	59	C	2	0	0	3	82	0	0	0	0
73	GSM275803	HER2-enriched	WU00431-16439PE_UU	73	C	4	1	0	3	44	1	0	0	1
74	GSM275800	HER2-enriched	WU00441-19793PE_UU	49	C	3	1	0	2	27	1	1	1	1
75	GSM275804	LumA	WU00509-19794PE_UU	57	C	2	1	0	3	51	0	0	0	0
76	GSM275805	HER2-enriched	WU00531-19795PE_UU	75	C	2	0	0	1	81	0	1	0	0
78	GSM275807	LumB	WU00556-21032PE_UU	46	C	2	1	0	2	88	0	1	1	0
82	GSM275810	Basal-like	WU00899-18760PE_UU	47	AA	2	0	0	3	83	0	1	1	0
86	GSM275813	Basal-like	WU01407-16456PE_UU	39	AA	2	0	0	3	80	0	0	0	0
88	GSM275815	HER2-enriched	WU01500-18755PE_UU	88	C	1	0	0	3	70	0	1	0	0
89	GSM275816	HER2-enriched	WU01502-16455PE_UU	74	C	2	2	0	1	88	0	1	0	0
90	GSM275817	HER2-enriched	WU01511-19773PE_UU	50	AA	1	1	0	3	82	0	0	0	1
91	GSM275818	LumA	WU01520-21957PE_UU	58	C	2	1	0	2	77	NA	1	1	NA
92	GSM275819	HER2-enriched	WU01540-14690PE_UU	46	C	3	1	0	3	20	1	NA	NA	0
93	GSM275799	LumB	WU01576-19797PE_UU	64	O	2	1	0	3	56	0	1	1	0
95	GSM275821	LumB	WU01587-16348PE_UU	72	C	1	0	1	2	82	0	1	0	0
96	GSM275822	LumB	WU01613-16349PE_UU	78	C	2	2	0	3	17	0	1	1	0
97	GSM275823	Basal-like	WU01680-16347PE_UU	47	C	2	0	0	3	77	0	0	0	1

TABLE 2-continued

Clinical and Subtype Data for Prototype Samples from Microarray/qRT-PCR Training Sets

Patient ID	GEO accession	Subtype Assignment (SigClust)	qPCR_name	Dx Age	Ethnicity	pT*	pN^	M	Grade%	Overall Survival	Vital Status	ER (IHC)**	PR (IHC)**	HER2 (status)^^
99	GSM275825	Basal-like	WU01790-16344PE_UU	32	AA	3	1	0	3	15	0	0	0	1
101	GSM275792	Basal-like	WU01887-16342PE_UU	73	AA	2	0	0	3	78	0	0	1	0
104	GSM275829	Basal-like	WU02104-16341PE_UU	57	C	2	1	0	3	51	0	0	0	0
105	GSM275830	Basal-like	WU02132-18761PE_UU	57	C	3	1	0	3	76	0	0	0	0
107	GSM275832	HER2-enriched	WU02338-21961PE_UU	42	C	2	1	0	3	59	1	1	1	1
108	GSM275833	Basal-like	WU02390-16330PE_UU	46	C	2	0	1	3	9	1	0	0	0
109	GSM275834	Basal-like	WU02455-14693PE_UU	44	C	3	0	0	3	15	1	0	0	0
110	GSM275797	HER2-enriched	WU02468-21279PE_UU	63	AA	1	2	0	3	72	0	NA	NA	1
113	GSM275795	HER2-enriched	WU02769-16337PE_UU	73	C	1	0	0	2	69	0	1	0	1
114	GSM275837	Basal-like	WU02771-14694PE_UU	43	C	3	0	0	3	16	1	0	0	1
116	GSM275839	Basal-like	WU02843-19762PE_UU	46	C	1	0	0	3	75	0	0	0	1
118	GSM275841	Basal-like	WU02948-16566PE_UU	44	C	1	0	0	3	62	0	0	0	0
120	GSM275842	HER2-enriched	WU03064-16462PE_UU	74	AA	2	0	0	3	70	0	1	1	1
121	GSM275843	Basal-like	WU03292-16446PE_UU	50	AA	3	0	0	3	79	0	0	0	0
123	GSM275791	HER2-enriched	WU03456-16361PE_UU	52	AA	1	0	0	3	67	0	1	1	0
125	GSM275846	LumB	WU03535-16451PE_UU	82	C	2	0	0	3	60	0	1	1	0
126	GSM275796	HER2-enriched	WU03653-16448PE_UU	49	C	1	2	0	3	102	0	0	0	1
127	GSM275847	Basal-like	WU03661-16447PE_UU	53	AA	4	1	0	3	3	1	1	1	0
128	GSM275788	LumA	WU03662-16452PE_UU	75	AA	2	0	0	3	45	0	0	0	0
129	GSM275848	Basal-like	WU03685-16502PE_UU	42	AA	2	0	0	3	65	0	0	0	0
131	GSM275850	Basal-like	WU03714-21262PE_UU	66	AA	1	0	0	3	72	0	1	1	0
132	GSM275793	HER2-enriched	WU03721-16570PE_UU	29	C	2	1	0	3	13	0	1	0	1
134	GSM275852	Basal-like	WU03791-16497PE_UU	61	C	1	1	0	3	62	0	0	0	0
135	GSM275853	Basal-like	WU03831-21959PE_UU	51	AA	2	1	0	3	68	0	0	0	1
139	GSM275857	Basal-like	WU03885-16469PE_UU	52	AA	2	1	0	3	26	1	1	1	0
140	GSM275858	HER2-enriched	WU03946-14842PE_UU	72	C	2	1	0	2	15	NA	0	1	1
141	GSM275789	Basal-like	WU04000-16466PE_UU	49	AA	1	2	0	3	24	1	NA	NA	0
144	GSM275861	HER2-enriched	WU04038-16465PE_UU	51	AA	2	1	0	2	62	0	1	1	1
146	GSM275863	Basal-like	WU04327-19803PE_UU	73	C	1	0	0	2	69	0	1	0	1
147	GSM275864	LumB	WU04532-16463PE_UU	75	AA	2	1	0	3	53	0	1	0	1
148	GSM275865	Basal-like	WU04834-16461PE_UU	42	AA	2	0	0	3	65	0	0	0	0
149	GSM275866	Basal-like	WU04952-19753PE_UU	64	AA	2	1	0	3	62	0	0	0	0
152	GSM275872	LumA	WU05094-16580PE_UU	29	C	1	1	0	1	60	0	1	1	0
153	GSM275873	HER2-enriched	WU05118-19759PE_UU	54	C	1	0	0	3	64	0	1	0	1
155	GSM275875	HER2-enriched	WU05162-21960PE_UU	43	C	2	0	0	3	95	1	NA	NA	NA

TABLE 2-continued

Clinical and Subtype Data for Prototype Samples from Microarray/qRT-PCR Training Sets

Patient ID	GEO accession	Subtype Assignment (SigClust)	qPCR_name	Dx Age	Ethnicity	pT*	pN^	M	Grade%	Overall Survival	Vital Status	ER (IHC)**	PR (IHC)**	HER2 (status)^^
156	GSM275876	Basal-like	WU05191-14791PE_UU	51	AA	4	1	1	2	46	1	NA	NA	1
157	GSM275877	HER2-enriched	WU05196-16573PE_UU	59	C	1	0	0	2	56	0	0	0	0
158	GSM275878	HER2-enriched	WU05207-16473PE_UU	72	AA	4	1	0	3	59	0	1	0	1
159	GSM275879	Basal-like	WU05215-16503PE_UU	79	AA	2	0	0	3	48	0	0	0	0
160	GSM275880	HER2-enriched	WU05337-14835PE_UU	51	C	2	1	0	3	14	1	0	0	0
161	GSM275881	HER2-enriched	WU05415-16357PE_UU	52	C	2	0	0	3	48	0	1	1	1
163	GSM275883	Basal-like	WU05478-16356PE_UU	41	C	2	0	0	3	54	0	0	0	0
165	GSM275885	Basal-like	WU05641-16354PE_UU	40	AA	2	0	0	3	54	0	0	0	0
167	GSM275887	Basal-like	WU05991-14687PE_UU	70	AA	4	1	0	3	19	1	0	0	0
168	GSM275888	Basal-like	WU06036-16352PE_UU	42	AA	2	0	0	3	50	0	0	0	NA
169	GSM275889	Basal-like	WU06397-19805PE_UU	64	C	2	0	0	3	54	0	0	0	0
170	GSM275890	HER2-enriched	WU06398-19767PE_UU	34	C	1	1	0	3	40	0	1	1	1
171	GSM275891	HER2-enriched	WU06416-19781PE_UU	74	C	1	0	0	3	51	0	0	0	0
172	GSM275892	LumA	WU06545-18758PE_UU	69	C	3	0	0	3	49	0	1	1	0
173	GSM275893	HER2-enriched	WU06559-14689PE_UU	42	AA	4	2	0	3	35	1	0	0	0
174	GSM275894	Basal-like	WU06580-16575PE_UU	44	AA	2	0	0	3	48	0	0	0	0
175	GSM275895	LumB	WU06611-16475PE_UU	46	C	2	0	0	3	51	0	1	1	1
177	GSM275897	Basal-like	WU06857-15260PE_UU	40	AA	2	1	0	3	11	1	0	0	0
178	GSM275898	LumA	WU07407-19770PE_UU	66	C	2	1	0	3	5	0	1	1	0
180	GSM275900	LumA	WU07509-19782PE_UU	57	AA	1	1	0	2	22	0	1	1	0
182	GSM275902	LumA	WU07512-14793PE_UU	83	C	1	NA	0	2	21	0	1	1	0
184	GSM275904	Basal-like	WU07558-16584PE_UU	41	AA	1	0	0	3	29	0	0	0	0
185	GSM275905	Basal-like	WU07589-16582PE_UU	70	AA	2	1	0	3	19	0	0	0	0
187	GSM275907	LumA	WU07791-19784PE_UU	78	C	1	0	0	2	53	0	1	1	1
188	GSM275908	LumA	WU07805-19777PE_UU	41	C	2	1	0	3	58	0	1	1	0
189	GSM275909	Basal-like	WU08626-16506PE_UU	34	AA	4	1	0	3	37	0	0	0	0
190	GSM34464	Basal-like	NA	61	NA	2	1	0	3	13	1	0	NA	NA
191	GSM140992	Basal-like	NA	29	NA	2	2	0	3	NA	1	0	NA	NA
192	GSM50148	Basal-like	NA	51	C	4	2	0	NA	74	1	0	NA	NA
193	GSM34562	Basal-like	NA	50	C	1	0	0	3	29	0	1	0	1
194	GSM52896	LumB	NA	79	NA	2	2	0	3	15	0	1	NA	1
195	GSM141067	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
196	GSM80240	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
197	GSM34547	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
198	GSM34483	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
199	GSM34482	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
200	GSM140990	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
201	GSM140991	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
202	GSM275777	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
203	GSM275778	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
204	GSM275781	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

TABLE 2-continued

Clinical and Subtype Data for Prototype Samples from Microarray/qRT-PCR Training Sets

Patient ID	GEO accession	Subtype Assignment (SigClust)	qPCR_name	Dx Age	Ethnicity	pT*	pN^	M	Grade%	Overall Survival	Vital Status	ER (IHC)**	PR (IHC)**	HER2 (status)^^
205	GSM275780	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
206	GSM275779	Normal-like	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*pathologic tumor stage: T1 ≤ 2 cm, T2 > 2 cm-5 cm, T3 > 5 cm, NA = not assessed

^pathologic node stage: N0 = no positive nodes, N1 = positive axillary nodes, NA = not assessed

% histological grade: 0 = grades 1&2, 1 = grade 3

**immunohistochemistry: 0 = no to moderate staining, 1 = strong staining in majority of cancer cells

^^immunonohistochemistry and fluorescence in-situ hybridization: 0 = negative by IHC (0, 1) or 2+ by IHC and negative by FISH, 1 = 3+ by IHC or 2+ by IHC and positive by FISH

TABLE 3

Clinical and Subtype Data for qRT-PCR Test Set

Patient ID	Subtype Prediction	pT*	pN^	Grade%	Overall Survival	Relapse Free Survival	Any Relapse***	DSS**	ER (IHC)^^	PR (IHC)^^	Her2 (IHC)^^
1001	LumB	2	1	1	2.3232877	0.690411	1	1	1	1	0
1002	Her2-enriched	2	NA	1	19.512329	2.9753425	1	1	0	0	0
1003	Her2-enriched	1	1	1	7.0410959	5.4	1	1	0	NA	1
1004	LumB	2	0	0	NA	NA	NA	1	1	1	0
1005	Her2-enriched	1	0	0	0.939726	0.3671233	1	1	0	NA	NA
1006	LumB	2	1	0	0.7178082	0.5178082	1	2	1	1	0
1007	LumB	NA	NA	0	3.3287671	1.7616438	1	1	1	1	0
1008	Her2-enriched	2	NA	0	0.8849315	0.7068493	1	1	1	0	0
1009	Basal-like	3	1	0	5.9917808	0.8246575	1	1	0	0	0
1010	LumA	2	1	NA	12.273973	9.0712379	1	1	1	1	NA
1011	Her2-enriched	2	1	1	3.2027397	1.2493151	1	1	0	0	1
1012	Normal-like	1	1	0	25.435616	22.709589	1	1	1	1	0
1013	LumB	2	1	0	NA	NA	NA	1	1	0	0
1014	LumB	2	0	0	16.654795	16.654795	0	2	1	1	0
1015	LumA	2	0	0	4.5150685	3.8821918	1	1	1	0	0
1016	Basal-like	2	0	1	2.0383562	1.6712329	1	1	0	0	0
1017	LumA	2	1	0	10.331507	5.9315068	1	1	1	0	0
1018	LumB	2	0	0	22.230137	21.89589	1	1	1	1	0
1019	LumB	2	1	0	3.0931507	1.4520548	1	1	1	1	0
1020	LumA	2	1	0	4.8630137	4.8630137	0	2	1	1	0
1021	LumA	1	0	0	6.7972603	4.9671233	1	2	1	1	0
1022	LumB	2	1	0	3.9150685	1.4164384	1	1	1	0	0
1023	LumA	2	1	0	25.945205	25.945205	0	3	1	1	0
1024	Basal-like	3	NA	1	2.4438356	1.7753425	1	1	0	0	0
1025	LumA	1	1	0	2.8767123	0.0027397	1	1	1	0	1
1026	LumA	1	NA	0	8.0821918	8.0821918	0	2	1	1	0
1027	LumB	1	1	0	25.778082	25.778082	0	3	1	1	0
1028	LumA	2	1	0	9.2520548	8.9753425	1	1	1	1	0
1029	Basal-like	2	0	1	3.4410959	1.9726027	1	1	0	0	0
1030	Her2-enriched	2	1	0	2.9232877	1.709589	1	1	1	1	1
1031	LumA	1	1	0	2.9616438	2.8958904	1	1	1	0	0
1032	LumA	2	0	0	4.509589	0.8465753	1	1	1	1	0
1033	LumB	1	NA	1	10.312329	9.9780822	1	1	NA	NA	NA
1034	LumB	1	0	1	15.19726	15.19726	0	2	1	1	0
1035	Basal-like	1	1	1	25.339726	25.339726	0	3	1	1	0
1036	LumA	2	1	0	3.4465753	1.460274	1	1	1	NA	0
1037	Basal-like	1	0	0	11.958904	11.958904	0	2	0	0	0
1038	Basal-like	2	1	1	2.4849315	2.2082192	1	1	0	NA	0
1039	LumA	2	NA	0	8.539726	6.8136986	1	1	1	1	0
1040	Basal-like	2	0	0	25.090411	25.090411	0	3	0	0	0
1041	LumA	2	1	0	3.7369863	1.7643836	1	1	NA	NA	0
1042	Basal-like	1	NA	0	2.1780822	0.9561644	1	1	1	1	1
1043	LumB	2	1	0	2.5452055	0.5232877	1	1	1	1	0
1044	LumA	1	1	0	2.630137	0.7315068	1	1	1	1	0
1045	Basal-like	2	1	1	1.4109589	1.060274	1	1	0	0	0
1046	Basal-like	2	1	1	24.835616	24.835616	0	3	0	0	0
1047	Basal-like	2	0	1	14.873973	14.536986	1	1	0	0	0
1048	Her2-enriched	1	1	1	2.3917808	1.5506849	1	1	1	1	0
1049	LumA	2	1	0	19.339726	19.339726	0	2	1	0	0
1050	LumB	2	1	0	13.605479	13.605479	0	2	1	0	NA
1051	LumB	2	1	0	2.4191781	1.4520548	1	1	1	1	0
1052	Basal-like	2	1	0	12.073973	11.739726	1	1	NA	NA	0

TABLE 3-continued

1053	Basal-like	2	1	0	0.3506849	0.0027397	1	1	0	0	0
1054	Basal-like	1	0	1	24.449315	24.449315	0	3	0	0	0
1055	LumB	2	0	0	6.1589041	4.1643836	1	1	1	0	0
1056	LumB	2	NA	0	1.2575342	0.0109589	1	1	1	1	0
1057	LumA	2	0	0	7.7753425	5.6630137	1	1	1	1	0
1058	Basal-like	1	1	1	24.323288	24.323288	0	3	0	NA	NA
1059	LumB	2	1	0	5.9863014	5.0876712	1	1	1	1	0
1060	LumB	1	1	1	24.115068	24.115068	0	3	1	1	0
1061	Basal-like	2	1	1	4.7972603	4.7972603	0	2	0	0	0
1062	Basal-like	3	1	1	24.084932	24.084932	0	3	0	0	0
1063	Basal-like	2	0	0	24.019178	24.019178	0	3	1	0	0
1064	Basal-like	2	0	1	22.791781	22.791781	0	3	0	NA	NA
1065	Her2-enriched	2	0	0	9.5150685	9.5150685	0	NA	0	0	0
1066	Basal-like	1	0	1	20.945205	20.945205	0	3	0	0	0
1067	Normal-like	2	0	0	10.917808	10.917808	0	2	NA	NA	1
1068	Her2-enriched	2	0	0	6.7013699	2.9726027	1	1	0	NA	0
1069	LumB	3	0	0	11.912329	11.912329	0	2	1	1	0
1070	LumB	2	0	0	20.731507	17.008219	1	3	1	1	0
1071	LumB	1	NA	0	4.0191781	1.2493151	1	2	1	1	0
1072	LumA	2	1	0	12.441096	12.441096	0	2	1	1	0
1073	Her2-enriched	3	0	0	20.660274	20.660274	0	3	NA	NA	1
1074	LumA	1	NA	0	4.7835616	4.4465753	1	1	1	0	0
1075	LumA	2	0	0	2.7534247	2.4246575	1	1	1	1	0
1076	LumA	2	1	0	20.539726	20.539726	0	3	1	NA	0
1077	LumA	1	1	0	20.408219	12.328767	1	3	1	1	0
1078	Normal-like	2	NA	0	2.6630137	1.2493151	1	1	1	NA	0
1079	Her2-enriched	1	1	0	NA	NA	NA	1	0	0	1
1080	Basal-like	2	1	0	NA	NA	NA	3	1	1	0
1081	Normal-like	2	1	0	NA	NA	NA	1	1	1	0
1082	Her2-enriched	2	0	1	NA	NA	NA	1	1	1	1
1083	LumB	2	0	0	NA	NA	NA	2	1	1	NA
1084	LumA	1	0	0	NA	NA	NA	3	NA	NA	0
1085	Basal-like	3	0	0	NA	NA	NA	1	1	0	0
1086	Basal-like	1	0	1	19.969863	19.969863	0	3	0	0	0
1087	Basal-like	1	0	0	19.657534	19.657534	0	3	0	0	0
1088	LumA	1	0	0	16.238356	3.9616438	1	1	1	1	0
1089	Her2-enriched	2	0	1	19.506849	19.506849	0	3	1	1	1
1090	LumA	1	0	0	8.5945205	6.9041096	1	1	0	0	0
1091	LumA	1	0	0	19.432877	2.6082192	1	3	1	1	0
1092	Basal-like	1	0	NA	19.405479	19.405479	0	3	0	NA	NA
1093	LumB	2	0	0	19.408219	16.756164	1	3	1	NA	0
1094	LumA	1	NA	0	19.358904	19.358904	0	3	1	1	0
1095	LumB	2	0	0	19.353425	19.353425	0	3	1	0	0
1096	LumA	2	0	0	13.345205	13.345205	0	2	1	1	0
1097	Her2-enriched	2	0	0	8.3890411	5.290411	1	2	1	0	0
1098	Her2-enriched	1	0	0	6.5863014	3.8767123	1	1	0	NA	1
1099	LumB	2	0	1	3.7780822	3.4438356	1	1	1	0	1
1100	Her2-enriched	1	0	0	19.20274	19.20274	0	3	1	0	1
1101	Basal-like	2	0	1	19.186301	19.186301	0	3	0	0	1
1102	Basal-like	1	0	0	5.8410959	4.7589041	1	1	0	NA	NA
1103	LumB	1	0	0	17.734247	3.6876712	1	2	1	NA	NA
1104	LumA	2	0	1	9.7917808	9.7917808	0	2	1	NA	0
1105	Basal-like	1	0	1	19.106849	19.106849	0	3	0	NA	0
1106	Basal-like	2	0	1	19.09863	19.09863	0	3	0	NA	NA
1107	LumA	1	0	0	19.871233	19.871233	0	3	1	1	0
1108	Basal-like	1	0	1	19.808219	19.808219	0	3	1	0	NA
1109	LumB	2	0	0	19.791781	1.6794521	1	3	1	1	0
1110	Her2-enriched	2	0	0	16.778082	16.778082	0	2	1	0	0
1111	LumA	1	0	0	19.789041	19.789041	0	3	1	1	0
1112	LumB	2	0	0	3.5068493	3.3643836	1	1	1	1	0
1113	LumB	2	0	1	2.3150685	0.9369863	1	2	1	0	0
1114	Basal-like	2	0	1	3.3232877	3.3232877	1	1	0	NA	0
1115	Her2-enriched	2	0	1	19.986301	19.986301	0	3	1	1	0
1116	Basal-like	2	0	1	NA	NA	NA	2	0	0	0
1117	Her2-enriched	1	0	1	19.758904	19.758904	0	3	1	NA	1
1118	LumB	1	0	1	19.717808	19.717808	0	3	1	1	0
1119	LumB	1	0	0	4.4054795	2.460274	1	1	1	NA	0
1120	LumB	2	0	1	5.3342466	4.8794521	1	1	1	1	0
1121	LumA	1	0	0	11.531507	11.531507	0	2	1	1	0
1122	Normal-like	1	0	0	15.424658	14.824658	1	2	NA	NA	NA
1123	LumB	2	0	0	3.8876712	3.8794521	1	1	1	1	NA
1124	Basal-like	1	0	0	19.252055	19.252055	0	3	0	0	0
1125	Normal-like	1	0	0	19.205479	19.205479	0	3	1	1	0
1126	LumB	1	0	0	20.005479	20.005479	0	3	1	1	0
1127	LumA	1	0	0	19.950685	19.950685	0	3	1	NA	0
1128	Normal-like	2	1	0	19.931507	2.2657534	1	3	1	NA	0
1129	LumA	1	0	0	19.849315	4.509589	1	3	1	1	0

TABLE 3-continued

1130	Basal-like	NA	0	1	8.0630137	8.0630137	0	NA	0	NA	NA
1131	Basal-like	2	1	1	1.030137	0.0520548	1	1	0	0	1
1132	Normal-like	1	0	0	19.608219	19.608219	0	3	1	1	0
1133	Her2-enriched	1	1	1	19.550685	19.550685	0	3	1	1	0
1134	Basal-like	2	1	0	2.0712329	1.230137	1	1	NA	NA	NA
1135	LumA	1	1	0	19.449315	19.449315	0	3	1	1	0
1136	LumB	1	1	0	5.0520548	4.4684932	1	1	1	1	0
1137	LumA	2	1	0	19.331507	17.657534	1	3	1	1	0
1138	LumA	1	1	0	19.331507	19.331507	0	3	1	1	0
1139	Basal-like	1	0	1	19.046575	19.046575	0	3	NA	0	0
1140	Her2-enriched	1	0	0	18.917808	18.917808	0	3	1	0	0
1141	LumB	3	NA	0	5.5205479	1.8410959	1	1	1	1	0
1142	LumA	1	0	0	18.649315	18.649315	0	NA	1	NA	0
1143	LumA	1	0	0	4.8876712	4.8876712	0	1	1	1	0
1144	Basal-like	NA	1	0	2.9972603	2.9123288	1	1	NA	NA	0
1145	LumA	1	0	0	18.753425	18.753425	0	3	1	1	NA
1146	LumA	3	NA	0	3.1917808	0.0027397	1	1	1	1	0
1147	Basal-like	1	0	1	10.79726	10.79726	0	2	1	0	0
1148	LumB	1	1	0	13.542466	4.6027397	1	2	1	1	0
1149	LumB	2	1	0	18.715068	18.715068	0	3	1	1	0
1150	Her2-enriched	2	1	1	2.6027397	2.0931507	1	1	0	NA	1
1151	LumB	NA	NA	1	18.641096	18.641096	0	3	1	1	0
1152	LumA	1	0	0	18.621918	18.621918	0	3	1	1	0
1153	LumA	NA	NA	0	1.460274	1.460274	0	NA	1	NA	0
1154	LumA	2	0	0	7.3479452	7.3479452	0	2	1	1	0
1155	LumA	1	1	0	7.4246575	6.939726	1	2	1	1	0
1156	Normal-like	2	0	0	18.452055	18.452055	0	3	0	0	0
1157	Her2-enriched	2	1	1	4.6246575	3.7671233	1	1	NA	0	NA
1158	LumA	1	1	0	7.3890411	8.060274	1	1	1	NA	0
1159	Her2-enriched	3	NA	1	18.986301	0.9863014	1	3	1	NA	1
1160	Her2-enriched	2	0	1	17.969863	17.969863	0	3	1	0	NA
1161	LumB	2	1	0	4.1452055	4.1452055	0	2	1	0	0
1162	LumA	1	1	0	17.909589	17.909589	0	3	1	1	0
1163	LumA	2	1	0	9.3972603	9.3972603	0	2	1	1	0
1164	LumA	2	0	0	7.8109589	7.8109589	0	2	1	1	0
1165	Her2-enriched	2	1	0	NA	NA	NA	1	0	0	NA
1166	Basal-like	1	0	1	17.378082	17.378082	0	3	0	0	0
1167	Her2-enriched	NA	NA	0	17.071233	17.071233	0	3	1	0	0
1168	LumA	1	0	NA	17.161644	17.161644	0	3	1	1	0
1169	Basal-like	3	NA	1	10.742466	6.5315068	1	1	0	NA	0
1170	Basal-like	1	NA	1	NA	NA	NA	1	0	0	0
1171	Her2-enriched	1	1	1	4.8438356	2.7506849	1	2	1	1	1
1172	LumB	1	NA	0	7.3972603	7.3972603	0	2	1	1	NA
1173	LumB	1	0	1	16.934247	3.4219178	1	3	1	1	0
1174	LumA	1	0	0	16.90137	16.90137	0	3	1	1	0
1175	LumB	2	1	0	16.882192	16.882192	0	3	1	1	0
1176	Her2-enriched	2	NA	1	9.1232877	9.1232877	0	2	1	1	1
1177	Basal-like	1	1	1	2.0986301	0.6547945	1	1	0	0	0
1178	Her2-enriched	2	0	1	2.1534247	1.7506849	1	1	0	0	0
1179	Basal-like	1	NA	1	0.0493151	0.0027397	1	1	0	0	0
1180	LumB	1	0	0	8.0328767	4.7917808	1	1	1	0	0
1181	LumA	1	0	0	7.8383562	7.8383562	0	2	1	NA	0
1182	Her2-enriched	3	1	0	16.706849	16.706849	0	3	0	0	0
1183	Basal-like	2	1	0	3.3835616	1.0547945	1	1	1	1	NA
1184	Basal-like	2	0	1	16.547945	16.547945	0	3	0	0	1
1185	Her2-enriched	2	0	0	16.520548	16.520548	0	3	1	1	1
1186	Normal-like	2	1	0	1.7506849	1.7506849	0	2	1	NA	0
1187	Her2-enriched	3	NA	0	1.7150685	0.0082192	1	1	NA	0	1
1188	Basal-like	2	1	1	16.479452	1.8219178	1	3	0	0	0
1189	Normal-like	2	0	0	11.153425	7.0520548	1	1	NA	NA	0
1190	LumA	2	0	0	16.287671	16.287671	0	3	1	1	0
1191	LumB	2	0	0	16.345205	16.345205	0	3	1	1	0
1192	LumB	1	0	0	16.227397	16.227397	0	3	1	NA	NA
1193	LumA	NA	NA	0	6.2821918	6.2821918	0	2	1	1	0
1194	LumA	3	NA	0	4.2767123	1.2849315	1	1	1	1	0
1195	LumA	2	0	0	9.7479452	9.7479452	0	2	1	1	NA
1196	LumB	1	NA	0	7.0684932	7.0684932	0	2	1	0	0
1197	LumA	1	0	0	5.8027397	5.8027397	0	2	1	0	0
1198	LumA	2	1	0	1.0383562	1.0383562	0	2	1	NA	0
1199	LumB	2	0	0	7.8520548	7.5178082	1	1	1	1	0
1200	LumA	2	0	0	15.863014	15.863014	0	3	1	1	0
1201	Normal-like	1	0	0	15.693151	15.693151	0	3	1	0	0
1202	LumB	1	NA	1	0.030137	0.030137	0	2	1	0	0
1203	Her2-enriched	2	1	0	10.046575	10.046575	0	2	0	0	0
1204	LumA	2	0	0	15.210959	15.210959	0	3	1	1	0
1205	LumA	1	NA	0	15.189041	10.890411	1	3	1	1	0
1206	LumA	2	0	0	15.169863	15.169863	0	3	1	NA	NA

TABLE 3-continued

1207	LumA	1	0	NA	2.2794521	0.9726027	1	1	1	NA	0
1208	Her2-enriched	1	0	1	8.0712329	6.0547945	1	1	0	0	1
1209	Basal-like	2	1	0	4.1287671	3.8630137	1	1	1	0	0
1210	LumB	1	0	0	15.035616	15.035616	0	3	1	1	0
1211	LumB	3	NA	0	1.9780822	0.9123288	1	1	1	1	0
1212	Basal-like	1	1	0	15.032877	15.032877	0	3	1	1	0
1213	Her2-enriched	2	1	0	15	15	0	3	0	0	0
1214	Basal-like	1	0	1	14.967123	14.967123	0	3	0	NA	0
1215	Normal-like	1	1	0	12.073973	12.073973	0	2	1	0	NA
1216	LumA	2	0	1	8.8	8.7835616	1	2	1	1	0
1217	LumA	1	1	0	14.89863	14.375342	1	3	1	1	0
1218	LumA	2	1	0	7.6767123	6.4410959	1	1	1	NA	0
1219	LumA	3	1	0	4.1890411	1.6493151	1	1	1	1	0
1220	Basal-like	3	1	0	2.5150685	0.4109589	1	1	0	0	0
1221	Normal-like	1	0	1	14.852055	14.852055	0	3	0	NA	NA
1222	LumA	1	0	0	14.772603	14.772603	0	3	NA	NA	0
1223	Basal-like	3	1	1	1.5589041	1.3260274	1	2	0	0	0
1224	Normal-like	1	0	0	14.709589	14.709589	0	3	NA	NA	0
1225	Basal-like	2	1	0	14.69589	13.29589	1	3	1	1	0
1226	LumA	3	NA	0	2.5232877	0.0410959	1	1	1	0	0
1227	LumA	1	0	0	14.578082	14.578082	0	3	1	0	0
1228	LumB	2	0	0	14.572603	14.572603	0	3	1	1	0
1229	Her2-enriched	2	0	1	14.613699	14.613699	0	3	0	0	0
1230	LumA	2	0	0	2.0109589	2.0109589	0	2	1	1	0
1231	LumA	1	0	0	14.542466	14.542466	0	3	1	0	0
1232	Her2-enriched	1	0	1	14.534247	14.534247	0	3	1	1	0
1233	LumB	1	NA	0	4.9123288	4.0849315	1	1	1	1	0
1234	LumB	NA	1	0	5.4876712	5.4876712	0	2	1	NA	0
1235	LumA	1	0	0	14.641096	14.641096	0	3	1	1	0
1236	LumA	2	0	0	14.520548	14.520548	0	3	1	1	0
1237	LumA	2	0	0	7.3780822	5.0109589	1	2	1	1	0
1238	Her2-enriched	3	NA	1	NA	NA	NA	1	1	0	0
1239	Her2-enriched	2	1	0	14.465753	14.465753	0	3	1	NA	NA
1240	Basal-like	3	1	0	14.438356	14.438356	0	3	1	NA	NA
1241	LumA	1	0	0	14.421918	14.421918	0	3	1	1	0
1242	LumA	2	0	0	14.419178	14.419178	0	3	1	NA	NA
1243	LumB	1	0	0	14.408219	14.408219	0	3	1	1	0
1244	LumB	1	1	0	10.013699	10.013699	0	2	1	1	0
1245	LumA	2	0	0	14.383562	14.383562	0	3	1	1	0
1246	LumB	2	0	0	4.5643836	4.5643836	0	2	1	1	0
1247	LumB	1	0	0	14.312329	1.4739726	1	3	1	1	0
1248	Normal-like	1	1	0	14.249315	14.249315	0	3	NA	NA	0
1249	LumA	3	0	0	13.49863	13.49863	0	2	1	0	0
1250	Basal-like	NA	0	1	14.235616	14.235616	0	3	0	NA	0
1251	LumB	1	0	0	14.945205	14.945205	0	3	1	0	0
1152	Normal-like	1	0	0	1.6547945	1.2493151	1	1	NA	NA	0
1253	LumA	2	1	0	4.21643841	4.2164384	0	2	1	0	0
1254	Normal-like	1	0	0	12.526027	12.526027	0	3	1	NA	0
1255	LumB	2	1	0	12.463014	12.463014	0	3	1	1	0
1256	Basal-like	2	1	0	3.7205479	1.7452055	1	1	1	1	NA
1257	Normal-like	1	0	0	12.427397	12.427397	0	3	1	NA	0
1258	LumA	2	0	0	12.372603	12.372603	0	3	1	NA	0
1259	LumA	1	1	0	12.328767	12.328767	0	3	1	NA	NA
1260	Basal-like	1	1	0	12.29589	2.5945205	1	3	1	1	0
1261	LumA	2	0	0	12.312329	12.312329	0	3	1	NA	0
1262	Normal-like	1	0	0	12.180822	12.180822	0	3	1	NA	0
1263	Basal-like	1	0	0	12.2	12.2	0	3	NA	NA	NA
1264	Her2-enriched	1	0	1	3.6	2.0684932	1	1	1	1	0
1265	LumA	2	1	0	12.2	12.2	0	3	1	1	0
1266	Normal-like	1	1	NA	11.857534	11.857534	0	3	1	NA	0
1267	Her2-enriched	2	0	1	11.186301	11.186301	0	3	0	0	0
1268	Normal-like	1	1	NA	11.073973	11.073973	0	3	1	NA	0
1269	Normal-like	3	0	0	10.969863	10.969863	0	3	1	1	0
1270	LumA	2	NA	0	10.920548	10.920548	0	3	1	1	0
1271	LumA	2	1	0	10.79726	10.79726	0	3	1	0	0
1272	LumB	1	0	0	10.668493	10.668493	0	3	1	0	0
1273	LumA	2	1	0	10.167123	10.167123	0	3	1	1	NA
1274	LumA	1	0	0	9.6821918	9.6821918	0	3	1	NA	0
1275	Normal-like	1	0	0	9.5917808	9.5917808	0	3	1	1	0
1276	Normal-like	1	0	0	9.6082192	9.6082192	0	3	1	1	0

TABLE 3-continued

1277	Basal-like	2	0	1	9.5287671	9.5287671	0	3	0	NA	0
1278	Normal-like	1	0	0	6.5835616	6.2547945	1	1	1	1	NA
1279	Basal-like	2	1	0	9.3643836	9.3643836	0	3	NA	NA	0

*tumor size: T1 ≤2 cm, T2 >2 cm-5 cm, T3 >5 cm, NA = not assessed

^nodal status: 0 = node negative, 1 = node positive, NA = nodal status unknown

%Nottingham histological grade: 0 = grades 1&2, 1 = grade 3, NA = unknown

***any relapse free survival: 0 = no relapse, 1 = relapse

**disease specific survival: 1 = death from breast CA, 2 = death from other than breast CA, 3 = alive, NA = unknown

^^immunohistochemistry

biomarker	0	1	NA
ER	<1% positive nuclei	≥1% positive nuclei	uninterpretable
PR	<1% positive nuclei	≥1% positive nuclei	uninterpretable
Her2-enriched	negative or weak expression	strong expression	uninterpretable

TABLE 4

Author	Sample	Platform	GEO Accessions (or other availability)	Use in Subtype Classification	Use in Risk Prediction	Number N-, no adjuvant systemic therapy	Number of Endocrine Therapy Only	% ER+
Parker et al	189	qRT-PCR		—	—	0	0	54%
Parker et al	279	qRT-PCR		Test	Test	0	0	62%
Parker et al	544	Agilent Custom, 1A, 1Av2	GSE10886	Common to qRT-PCR for Training (189): other in Test (355)	355 in Test	31	27	56%
Hess et al	133	Affymetrix U133A	bioinformatics.mdanderson.org/pubdata	Test	Test	0	0	62%
Ivshina et al	289	Affymetrix U133A	GSE4922	Test	Test	142	66	86%
Loi et al	414	Affymetrix U133A & U133 + 2	GSE6532	Test	Test	137	277	89%
van de Vijver et al	295	Agilent	GSE2845	Test	Untreated for Training (165); others in Test (130)	165	20	76%
Wang et al	286	Affymetrix U133A	GSE2034	Test	Test	286	0	73%

TABLE 5

Multivariate and univariate analyses using 1451 samples from a combined microarray test set with clinical data

	Univariate		Multivariate* (subtype)		Multivariate* (clinical)		Multivariate*†‡ (subtype + clinical)	
Variable	Co-efficient	p-value^	Coefficient	p-value^	Coefficient	p-value^	Coefficient	p-value^
Basal-like	0.14	0.25	0.12	5.10E−01	—		−0.11	5.50E−01
HER-enriched	0.62	1.00E−08	0.53	1.60E−03	—		0.35	4.00E−02
LumA	−0.94	1.00E−22	−0.67	6.20E−05	—		−0.64	1.60E−04
LumB	0.42	5.60E−06	0.3	5.50E−02	—		0.24	1.30E−01
ER Status	−0.47	1.80E−06	—	—	−0.5	5.50E−07	−0.37	3.00E−03
Tumor Size	0.62	3.50E−12	—	—	0.54	6.10E−09	0.47	5.30E−07
Node Status	0.37	2.80E−05	—	—	0.24	1.10E−02	0.19	5.00E−02

*Normal-like class used as reference state

^Significant variables are in italics

†p = 4e−10 (by the likelihood ratio test) for comparison with the Subtype model

‡= 2e−13 (by the likelihood ratio test) for comparison with the Clinical model

TABLE 6

Distribution of Intrinsic Subtypes by ER-status

Test Set	ER-status	# Samples	% LumA	% LumB	% HER2-enriched	% Basal-like	% Normal-like
UNC	ER-positive	137	44%	35%	7%	4%	9%
	ER-negative	107	7%	5%	19%	51%	18%
Hess et al	ER-positive	82	44%	32%	10%	1%	13%
	ER-negative	51	2%	2%	41%	51%	4%
Ivshina et al	ER-positive	211	42%	29%	11%	8%	9%
	ER-negative	34	9%	15%	35%	38%	3%
Loi et al	ER-positive	349	39%	38%	8%	7%	8%
	ER-negative	45	18%	9%	33%	27%	13%
van de Vijver et al	ER-positive	225	39%	31%	14%	4%	12%
	ER-negative	70	1%	0%	31%	64%	3%
Wang et al	ER-positive	209	35%	33%	11%	8%	13%
	ER-negative	77	5%	3%	29%	57%	6%

TABLE 7

T/FAC pathological complete response rates for PAM50 subtypes and triple-negative classification

Classification	RD	pCR
Basal-like	11 (41%)	16 (59%)
HER2-enriched	17 (59%)	12 (41%)
LumA	36 (100%)	0 (0%)
LumB	22 (82%)	5 (18%)
Normal-like	13 (93%)	1 (7%)
Triple Negative	13 (50%)	13 (50%)
Any positive	82 (80%)	20 (20%)
Triple Negative/Basal	6 (35%)	11 (65%)
Triple Negative/Non-Basal	7 (78%)	2 (22%)
Non-Triple Negative/Basal	4 (50%)	4 (50%)
Non-Triple Negative/Non-Basal	78 (83%)	16 (17%)

*Percentages are calculated by the total per classification

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Example 2

Introduction and Background Data

[0138] This technology also covers the use of the PAM50-based intrinsic subtype classifier as a predictive and prognostic signature in the neoadjuvant endocrine therapy setting. Postmenopausal patients with Stage 2 and 3 ER and/or PgR positive breast cancer can be treated with an endocrine agent, typically an aromatase inhibitor or tamoxifen, before surgery to improve clinical outcomes, i.e., to promote the use of breast conserving surgery or to improve operability in the setting of a tumor that has invaded into the tissues surrounding the breast. A predictive test to increase the confidence that an individual patient will respond to neoadjuvant endocrine therapy is a significant advance.

Summary

[0139] The PAM50 based intrinsic subtype and proliferation-weighted risk score, when applied to samples from ER+ breast cancers harvested after initiating treatment with an endocrine agent, can be used to predict response to neoadjuvant endocrine therapy and determine the prognosis for patients with ER+ breast cancer who will undergo long term therapy with an endocrine agent. A prognostic gene expression model trained on tumor samples taken before treatment (PAM50 proliferation weighted risk score—described elsewhere herein) was applied to samples taken after the initiation of neoadjuvant endocrine therapy. This approach is unique because previous studies on the interaction of gene expression profiles and prognosis have only examined pre-treatment samples and have never applied these models to post treatment samples. The prognostic and predictive properties of the PAM50 intrinsic subtype and proliferation weighted prognostic model in baseline samples is compared to the same models applied to samples taken one month after initiating neoadjuvant endocrine therapy. Application of the PAM50 intrinsic subtype and proliferation-weighted risk of relapse model to the one month on treatment samples accurately identifies aggressive tumors that fail to respond to neo adjuvant or adjuvant endocrine treatment. Patients with these tumors should be immediately triaged to alternative neoadjuvant treatments, such as chemotherapy, because these poor tumors behave as endocrine therapy refractory aggressive disease. A high degree of correlation was established between the Ki67 proliferation marker and the proliferation weighted PAM50 risk score supporting the claim that the PAM50 proliferation weighted risk score has prognostic properties. However these prognostic properties are markedly enhanced when the analysis is applied to samples harvested from tumors that have been exposed to an endocrine agent. In practice this can be easily achieved by prescribing an endocrine agent for a few weeks before definitive surgery or by re-sampling a tumor early in the course of neoadjuvant endocrine treatment in order to identify unresponsive tumors.

Methodology:

[0140] The evidence to support these claims arises from a National Cancer Institute sponsored Phase 2 trial of neoadjuvant therapy with the aromatase inhibitor letrozole (NCI Grant No. RO1 CA095614). Eligibility for the trial required postmenopausal women with ER and/or PgR positive Stage 2 and 3 breast cancers. Patients received 4 months of therapy and then they underwent surgery. Frozen tumor samples were obtained at baseline, one month and at surgery. The samples were analyzed by frozen-section and RNA was extracted using standard methodologies from tumor rich specimens and subjected to gene expression analysis using Agilent 1×44K arrays. The data was normalized to the data set used to train the PAM50 classifier (methods described above) and two readouts were produced: An intrinsic classification (LumA, LumB, HER2-enriched, Basal-like and Normal-like) and a proliferation weighted PAM50 risk score. The aim of this study was to correlate the outcomes of neoadjuvant endocrine therapy with the intrinsic classification and the proliferation weighted risk score derived from both the baseline sample and the on treatment sample taken at one month.

Results:

[0141] The PAM50 intrinsic subtype and proliferation weighted risk score showed marked changes at one month post therapy (Table 8). Most of the transitions occurred in the LumB group with the majority shifting to LumA, but 16% remained in the LumB category despite treatment. In contrast, most LumA tumors stayed LumA post therapy. These transitions were due to the suppression of the proliferation cluster in the LumB group since the PAM50 proliferation weighted risk score showed similar shifts, with the majority of tumors typed high risk (68%) becoming intermediate or low risk in the on treatment samples. Tight correlation with Ki67 immunohistochemistry further underscores this conclusion. The correlation between baseline Ki67 values and PAM50 proliferation weighted risk score was high (P=2.8×E-8). Similarly the one month Ki67 values and the one month PAM50 proliferation score were also tightly correlated (P=3.8E-10). However, while the baseline PAM50 proliferation weighted risk score subtype exhibited only a very weak correlation with the end of study Ki67 values (P=0.04), there was a tight correlation between the one month PAM50 proliferation weighted risk score and the end of study Ki67 values—most of which were obtained at surgery 4 to 6 months later (P=6.8E-11). This last observation strongly supports the claim that an early on treatment PAM50 based test can be used to predict whether the final surgical samples will have favorable biomarker features, such as a low proliferation rate.

[0142] To determine the clinical correlations associated with these endocrine-therapy induced changes in intrinsic breast cancer subtype and risk score, four endpoints were examined: clinical response (RECIST criteria), pathological T size (T1 versus higher—as evidence for pathological down staging with treatment), dichotomized Ki67 values (with tumors exhibiting a Ki67 natural log value of 1 or less considered to be exhibiting a favorable profile) and relapse events. The baseline subtype or risk score showed no convincing ability to predict any of these endpoints, which, in terms of the relapse, is likely a function of the small sample size in this trial (Table 9). In contrast, and despite the small sample size, the PAM50 intrinsic subtype at one month (Table 10) did show statistically significant relationships with clinical response (P=0.01), favorable end of treatment Ki67 value (P=0.0003) and relapse (0.009). These strong relationships were driven by the extremely poor outcome associated with tumors that were either designated "non-luminal" or Luminal B in the on treatment specimens. The PAM50 proliferation-weighted risk score had similar properties. Baseline PAM50 proliferation-weighted risks score did not predict the neoadjuvant or long term outcomes very effectively (Table 11). However tumors that were designated high risk at one month showed significant correlations with poor outcomes in all four endpoints examined, i.e., poor clinical response (P=0.02), low pathological downstaging (p=0.02), unfavorable end of treatment Ki67 value (P=0.0001) and relapse (p=0.001) (Table 12).

[0143] Thus, application of the PAM50 based intrinsic subtype and risk score to tumor samples harvested from primary ER+ breast cancers undergoing presurgical treatment with an endocrine agent can be used for the following purposes:

[0144] 1) Prediction of a failure to respond to neoadjuvant endocrine therapy

[0145] 2) Determination of the prognosis for patients with ER+ breast cancer subsequently undergoing adjuvant endocrine treatment.

TABLE 8

PAM50 subtype and proliferation-weighted risk group switching at one month after treatment.

Change Category	Number	Percentage
PAM50 Intrinsic Subtype Changes		
LumA to LumA	18	31.0
LumA to LumB	1	1.7
LumA to Non-Lum	0	0
LumB to LumA	29	50.0
LumB to LumB	6	10.3
LumB to Non-Lum	1	1.7
Non-Lum to Non-Lum	1	1.7
Non Lum to LumA	0	0
Non Lum to LumB	2	3.4
Total	58	100
Proliferation weighted PAM50 Risk Score		
Low to Low	5	8.6
Low to Med	1	1.7
Low to High	0	0
Med to Low	7	12.1
Med to Med	12	20.7
Med to High	1	1.7
High to Low	11	19
High to Med	14	24.1
High to High	7	12.1
Total	58	100

TABLE 9

Interactions between the baseline PAM50 intrinsic subtype designations and outcomes from neoadjuvant endocrine therapy.

Subtype or score at Baseline	End of Study Endpoint	Number/Total	% favorable outcome	P value on interaction
Subtype	Clinical Response CR + PR v SD + PD			0.54
LumA		28/76	60.71	
LumB		42/76	69.05	
NonLum†		6/76	50.00	
	Path tumor size* ≤2 cm versus >2 cm			0.29
LumA		29/78	37.79	
LumB		43/78	48.84	
NonLum†		6/78	16.67	
	Log normal Ki67# ≤ log 1.0 versus >1.0			0.03
LumA		30/29	66.67	
LumB		43/79	37.21	
NonLum†		6/79	33.33	
	Relapse Yes versus No			0.262
LumA		30/78	90.00	
LumB		42/78	90.4762	
NonLum†		6/78	66.67	

*Since all patients had clinical stage 2 or 3 disease, pathological tumor stage one are surgery was taken as evidence of successful down-staging. Tumors that progressed during therapy and underwent neoadjuvant chemotherapy are assumed to have a pathological T size of greater than 2 cm at the end of study.
#End of study Ki67 is defined as either the surgical specimen or the one month value if the patient progressed on neoadjuvant endocrine therapy and underwent chemotherapy or did not undergo surgery.
†Non-Luminal refers to samples designated Basal-like or HER2 enriched. Normal-like is not included in this analysis because these samples are assumed to not contain sufficient tumor cells for adequate subtyping.

TABLE 10

Interactions between one month on treatment PAM50 intrinsic subtype designations and outcomes from neoadjuvant endocrine therapy.

PAM50 Subtype at one month	End of Study Endpoint	Number/Total	% favorable outcome	P value on interaction
Subtype	Clinical Response CR + PR v SD + PD			0.01
LumA		45/56	75.56	
LumB		9/56	44.44	
NonLum		2/56	0	
	Path tumor size* ≤2 cm versus >2 cm			0.41
LumA		46/57	47.83	
LumB		9/57	22.22	
NonLum		2/57	50.00	
	Log normal Ki67# ≤ log 1.0 versus >1.0			0.0003
LumA		47/58	61.70	
LumB		9/58	0	
NonLum		2/58	0	
	Relapse Yes versus No			0.009
LumA		45/53	93.62	
LumB		7/53	57.14	
NonLum		2/53	50.00	

*Since all patients had clinical stage 2 or 3 disease, pathological tumor stage one are surgery was taken as evidence of successful down-staging. Tumors that progressed during therapy and underwent neoadjuvant chemotherapy are assumed to have a pathological T size of greater than 2 cm at the end of study.
#End of study Ki67 is defined as either the surgical specimen or the one month value if the patient progressed on neoadjuvant endocrine therapy and underwent chemotherapy or did not undergo surgery.
†Non-Luminal refers to samples designated Basal-like or HER2 enriched. Normal-like is not included in this analysis because these samples are assumed to not contain sufficient tumor cells for adequate subtyping.

TABLE 11

Interactions between baseline PAM50 proliferation weighted risk score designations and outcomes from neoadjuvant endocrine therapy

Risk Score, with proliferation at Baseline	End of Study Endpoint	Number/Total	% favorable outcome	P value on interaction†
	Clinical Response CR + PR v SD + PD			0.4573
Low		9/76	44.44	
Med		28/76	67.79	
High		39/76	66.67	
	Path tumor size* ≤2 cm versus >2 cm			1.0
Low		9/78	44.44	
Med		29/78	41.38	
High		37/78	42.50	
	Log normal Ki67# ≤log 1.0 versus >1.0			0.03431
Low		9/79	77.78	
Med		30/79	56.67	
High		40/79	35.00	
	Relapse Yes versus No			0.1191
Low		9/74	77.78	
Med		29/74	96.67	
High		36/74	84.62	

*Since all patients had clinical stage 2 or 3 disease, pathological tumor stage one are surgery was taken as evidence of successful down-staging. Tumors that progressed during therapy and underwent neoadjuvant chemotherapy are assumed to have a pathological T size of greater than 2 cm at the end of study.
#End of study Ki67 is defined as either the surgical specimen or the one month value if the patient progressed on neoadjuvant endocrine therapy and underwent chemotherapy or did not undergo surgery.
†Non-Luminal refers to samples designated Basal-like or HER2 enriched. Normal-like is not included in this analysis because these samples are assumed to not contain sufficient tumor cells for adequate subtyping.

TABLE 12

Interactions between one month on therapy PAM50 proliferation weighted risk score and outcomes from neoadjuvant endocrine therapy

PAM50 proliferation weighted risk score at one month	End of Study Endpoint	Number/Total	% favorable outcome	P value on interaction
	Clinical Response CR + PR v SD + PD			0.02

TABLE 12-continued

Interactions between one month on therapy PAM50 proliferation weighted risk score and outcomes from neoadjuvant endocrine therapy

PAM50 proliferation weighted risk score at one month	End of Study Endpoint	Number/Total	% favorable outcome	P value on interaction
Low		21/56	80.95	
Med		27/56	70.37	
High		8/56	25.00	
	Path tumor size* ≤2 cm versus >2 cm			0.02
Low		23/57	47.83	
Med		26/57	53.85	
High		8/57	0	
	Log normal Ki67# ≤ log 1.0 versus >1.0			0.0001
Low		23/58	78.26	
Med		27/58	40.74	
High		8/58	0	
	Relapse Yes versus No			0.001
Low		23/56	95.65	
Med		27/56	92.59	
High		6/56	33.33	

*Since all patients had clinical stage 2 or 3 disease, pathological tumor stage one are surgery was taken as evidence of successful down-staging. Tumors that progressed during therapy and underwent neoadjuvant chemotherapy are assumed to have a pathological T size of greater than 2 cm at the end of study.
#End of study Ki67 is defined as either the surgical specimen or the one month value if the patient progressed on neoadjuvant endocrine therapy and underwent chemotherapy or did not undergo surgery.
†Non-Luminal refers to samples designated Basal-like or HER2-like, Normal-like is not included in this analysis because these samples are assumed to not contain sufficient tumor cells for adequate subtyping.

Example 3

[0146] A risk of relapse analysis was performed on the samples described in Example 1, except the normal-like class was removed from the model. The normal-like class was represented using true "normals" from reduction mammoplasty or grossly uninvolved tissue. Thus, this class has been removed from the all outcome analyses and this classification is considered as a quality-control measure. Methods not described below are identical to the methods described in Example 1.

Methods

Prognostic and Predictive Models Using Clinical and Molecular Subtype Data:

[0147] Univariate and multivariate analyses were used to determine the significance of the intrinsic substypes (LumA, LumB, HER2-enriched, and basal-like) in untreated patients and in patients receiving neoadjuvant chemotherapy. For prognosis, subtypes were compared with standard clinical variables (T, N, ER status, and histological grade), with time to relapse (i.e., any event) as the end point. Subtypes were compared with grade and molecular markers (ER, progesterone receptor (PR), HER2) for prediction in the neoadjuvant setting because pathologic staging is not applicable. Likelihood ratio tests were done to compared models of available clinical data, subtype data, and combined clinical and molecular variables. Categoric survival analyses were performed using a log-rank test and visualized with Kaplan-Meier plots.

Developing Risk Models with Clinical and Molecular Data

[0148] The subtype risk model was trained with a multivariate Cox model using Ridge regression fit to the node-negative, untreated subset of the van de Vijver et al. (2002) cohort. A ROR score was assigned to each test case using correlation to the subtype alone (1) (ROR-S) or using subtype correlation along with tumor size (2) (ROR-C):

$$ROR\text{-}S=0.05*\text{Basal}+0.12*\text{Her2}+-0.34*\text{Lum}A+0.0.23*\text{Lum}B \quad (1)$$

$$ROR\text{-}C=0.05*\text{Basal}+0.11*\text{Her2}+-0.23*\text{Lum}A+0.09*\text{Lum}B+0.17*T \quad (2)$$

[0149] The sum of the coefficients from the Cox model is the ROR score for each patient. The classify samples into specific risk groups, thresholds were chosen from the training set as described in Example 1. SiZer analysis was performed to characterize the relationship between the ROR score and relapse-free survival. The 95% CIs for the ROR score are local versions of binomial CIs, with the local sample size computed from a Gaussian kernel density estimator based on the Sheather-Jones choice of window width.

Comparison of Relapse Prediction Models

[0150] Four models were compared for prediction of relapse: (1) a model of clinical variables alone (tumor size, grade, and ER status), (2) ROR-S, (3) ROR-C, and (4) a model combinding subtype, tumor size, and grade. The C-index was chose to compare the strength of the various models. For each model, the C-index was estimated from 100 randomizations of the untreated cohort into two-thirds training set and one-thirds test set. The C-index was calculated for each test set to form the estimate of each model, and C-index estimates were compared across models using the two sample t test.

Results

Risk of Relapse Models for Prognosis in Node-Negative Breast Cancer

[0151] Cox models were tested using intrinsic subtype alone and together with clinical variables. Table 13 shows the multivariable analyses of these models in an independent cohort of untreated patients (see Example 1). In model A, subtypes, tumor size (T1 or greater) and histologic grade were found to be significant factors for ROR. The great majority of basal-like tumors (95.9%) were found to be medium or high grade, and therefore, in model B, which is an analysis without grade, basal-like becomes significant. Model C shows the significance of the subtypes in the node-negative population. All models that included subtype and clinical variables were significantly better than either clinical alone (P<0.0001) or subtype alone (P<0.0001). A relapse classifier was trained to predict outcomes within the context of the intrinsic subtypes and clinical variables. A node-negative, no systemic treatment cohort (n=141) was selected from the van de Vijver et al. (2002) microarray data set to train the ROR model and to select cut-offs. There was a clear improvement in preduction with subtype (ROR-S) relative to the model of available clinical variables only (see Parker et al. (2009) J Clin Oncol 27(8):1160-1167). A combination of clinical variables and subtype (ROR-C) is also a significant improvement over either individual predictor. However, information on grade did not significantly improve the C-index in the combined model, indicating that the prognostic value of grade had been superseded by information provided by the intrinsic subtype model. When using ROR-C for ROR in a prognostic test set of untreated node-negative patients, only the LumA group contained any low-risk patients, and the three-class distinction of low, medium, and high risk was prognostic. Also, ROR-C scores have a linear relationship with probability of relapse at 5 years.

TABLE 13

Models of relapse-free survival (untreated)

	Model A		Model B		Model C	
Variable	Hazard ratio	P	Hazard ratio	P	Hazard ratio	P
Basal-like*	1.33	0.33	1.79	0.3	1.58	0.066
HER-enriched*	2.53	0.00012	3.25	<0.0001	2.9	<0.0001
LumB*	2.43	<.0001	2.88	<0.0001	2.54	<0.0001
ER Status†	0.83	0.38	0.83	0.34	0.83	0.32
Tumor Size‡	1.36	0.034	1.43	0.012	1.57	0.001
Node Status§	1.75	0.035	1.72	0.041	—	—
Histologic grade||	1.4	0.0042	—	—	—	—
Full v. subtype¶		<.0001		<0.0001		<0.0001

TABLE 13-continued

Models of relapse-free survival (untreated)

	Model A		Model B		Model C	
Variable	Hazard ratio	P	Hazard ratio	P	Hazard ratio	P
Full v. clinical#		<.0001		<0.0001		<0.0001

*Luminal A class used as reference state in multivariable analysis.

†Hazard ratios for ER using positive marker in the numerator.

‡Size ≤2 cm versus >2 cm.

§Any positive node.

||Grade encoded as an ordinal variable with three levels

¶Significant P values indicate improved prediction relative to subtype alone.

#Significant P values indicate improved prediction relative to clinical data alone.

Subtypes and Prediction of Response to Neoadjuvant T/FAC Treatment

[0152] The Hess et al. (2006) study that performed microarray on tumors from patients treated with T/FAC allowed investigation of the relationship between the subtypes and clinical markers and how each relates to pCR>. Table 14 shows the multivariable analyses of the subtypes together with clinical molecular markers (ER. PR, HER2) and either with (model A) or without (model B) histologic grade. The only significant variables in the context of this study were the intrinsic subtypes. A 94% sensitivity and 97% negative predictive value was found for identifying nonresponders to chemotherapy when using the ROR-S model to predict pCR. The relationship between high-risk scores and a higher probability of pCR is consistent with the conclusion that indolent ER-positive tumors (LumA) are less responsive to chemotherapy. However, unlike ROR for prognosis, a plateau seems to be reached for the ROR versus probability of pCR, confirming the presence of significant chemotherapy resistance among the highest risk tumors.

TABLE 14

Models of neoadjuvant response

	Model A		Model B		Model C	
Variable	Odds ratio	P	Odds ratio	P	Odds ratio	P
Basal-like*	1.33	0.33	1.79	0.3	1.58	0.066
HER-enriched*	2.53	0.00012	3.25	<0.0001	2.9	<0.0001
LumB*	2.43	<.0001	2.88	<0.0001	2.54	<0.0001
ER Status†	0.83	0.38	0.83	0.34	0.83	0.32
PR Status†	1.36	0.034	1.43	0.012	1.57	0.001
Histologic grade‡	1.4	0.0042	—	—	—	—
Full v. subtype§		<.0001		<0.0001		<0.0001
Full v. clinical||		<.0001		<0.0001		<0.0001

*Luminal A class used as reference state in multivariable analysis.

†Hazard ratios for ER, PR and HER2 are positive marker in the numerator.

‡Grade encoded as an ordinal variable with three levels.

§Significant P values indicate improved prediction relative to subtype alone.

||Significant P values indicate improved prediction relative to clinical data alone.

Example 4

[0153] In this study, qRT-PCR and previously established cut points (see Example 1) was used to assess the prognostic value of the PAM50 classifier in the common, clinically-important group of women who are estrogen receptor positive and treated with tamoxifen as their sole adjuvant systemic therapy. Unlike in most previous reports, this homogeneously-treated study cohort includes a large proportion of lymph node positive patients. The available detailed long term follow-up permits assessment not only of relapse-free survival, but also of the risk of breast cancer disease-specific death, in comparison with all standard clinicopathologic risk factors.

Methods

Patients:

[0154] The study cohort is derived from female patients with invasive breast cancer, newly diagnosed in the province of British Columbia in the period between 1986 and 1992. Tissue had been excised at various hospitals around the province, frozen and shipped to the central estrogen receptor (ER) laboratory at Vancouver Hospital; the portion of the received material that was formalin-fixed and paraffin-embedded as a histological reference is used in this study. Clinical information linked to the specimens includes age, histology, grade, tumor size, number involved axillary nodes, lymphatic or vascular invasion, ER status by the DCC method, type of local and initial adjuvant systemic therapy, dates of diagnosis, first local, regional or distant recurrence, date and cause of death. Characteristics of this patient cohort have been previously described in detail in a population-based study validating the prognostic model ADJUVANT! [Olivotto 2005], and the same source blocks were used to assemble tissue microarrays that have been characterized for ER [Cheang 2006] and HER2[Chia 2008] expression. For this study, patients were selected who had ER positive tumors by immunohistochemistry, and received tamoxifen as their sole adjuvant systemic therapy. During the time period when these patients received their treatment, provincial guidelines recommended adjuvant tamoxifen for post-menopausal women, with ER-positive tumors who had some high risk features present such as lymphovascular invasion. Similar patients without high risk features were mainly treated without adjuvant systemic therapy. In most cases, chemotherapy was only offered to premenopausal women.

RNA Preparation:

[0155] RNA was isolated from pathologist-guided tissue cores. Briefly, H&E sections from each block were reviewed by a pathologist. Areas containing representative invasive breast carcinoma were selected and circled on the source block. Using a 1.0 mm punch needle, at least two tumor cores were extracted from the circled area. RNA was recovered using the High Pure RNA Paraffin Kit (Roche Applied Science, Indianapolis Ind.), DNA removed with Turbo Dnase (Ambion, Austin Tex.), and RNA yield assessed using an ND-1000 Spectrophotometer (Nanoprop Technologies, Rockland Del.).

qRT-PCR:

[0156] cDNA synthesis was done using a mixture of random hexamers and gene-specific primers, and qPCR was performed with the Roche LightCycler 480 instrument as previously described [Mullins 2007]. Each 384-well plate contained samples in duplicate (2.5 ng cDNA per reaction) and a calibrator in triplicate (10 ng cDNA per reaction). A tumor sample was considered of insufficient quality if any of the reference controls (ACTB, PSMC4, RPLP0, MRPL19, or SF3A1) failed. PCR was technically successful for all 50 discriminator genes in 73% of cases, and for 49 of the 50 in another 15% of cases. To assess the tolerance of the PAM50 assay results to missing gene information, ROR-C values were assessed in the data following random simulated removal of an increasing number of genes. Loss of one gene resulted in a 0-2 unit change in risk score, corresponding to a 1% increase/decrease in disease-specific survival at 10 years.

Assignment of Biological Subtype to Clinical Samples:

[0157] Gene expression centroids corresponding to Luminal A, Luminal B, HER2-enriched, Basal-like and Normal-like subtypes were constructed using the intrinsic 50 gene panel as described in Example 1 and in Parker et al. (2007 J. Clin. Oncol. 27(8):1160-7, which is herein incorporated by reference in its entirety). Specimens were assigned to an intrinsic subtype based on the nearest centroid distance calculated by Spearman's rank correlation, by investigators blinded to outcome data.

Relation of PAM50 Subtype to Clinical Outcome:

[0158] Statistical analyses were conducted using SPSS v16.0 and R v2.8.0. Univariate analysis of tumor subtype against breast cancer distant relapse-free and breast cancer disease-specific survival was performed by Kaplan-Meier analysis, with log rank test for significance. Multivariate analysis was performed against the standard clinical parameters of tumor size, nodal status (% positive nodes over total examined), histologic grade, patient age and HER2 status (based on adjacent cores from the same source block, assembled into tissue microarrays and subjected to immunostaining and FISH analysis using clinical-equivalent protocols [Chia 2008]). Cox regression models [Cox 1984] were built to estimate the adjusted hazard ratios of the qPCR-assigned breast cancer subtypes[Truong 2005]. Only cases with information for all the covariates were included in the analysis. Smoothed plots of weighted Schoenfeld residuals were used to assess proportional hazard assumptions [Grambsch 1994].

Relation of Risk-Of-Relapse (ROR) Score to Clinical Outcome:

[0159] The ROR score algorithm (ROR-S incorporating a sample's correlation to the Luminal A, Luminal B, HER2-enriched, and Basal-like subtypes; ROR-C incorporating this information plus tumor size) was trained and validated on three microarray-profiled and one qPCR-profiled breast cancer series. Risk stratification cutpoints were assigned in the training set such that no Luminal A patients fell into the high risk category, and no Basal-like patients fell into the low risk category. Kaplan-Meier and Cox regression analyses were conducted as above.

Results

[0160] From surgical specimens which had been formalin-fixed and paraffin embedded 15-20 years previously, tumor cores were extracted from pathologist-identified areas of invasive breast carcinoma for 991 cases. Following RNA extraction, 815 samples yielded at least 1.2 μg total RNA at a concentration of at least 25 ng/μL, and proceeded to PCR analysis. Template was of technically sufficient quality (based on internal housekeeper gene controls) for qRT-PCR in 806. Among these cases, a total of 711 specimens yielded high quality qRT-PCR quantitative data for at least 49 of the PAM50 discriminator genes, and were included in subsequent clinical and survival analyses. Clinical characteristics for these 711 patients are presented in Table 15.

TABLE 15

Clinical Parameter		Whole TAM series	Luminal A	Luminal B	Her2	Basal	Normal
Sample Size	N	711	329	312	58	3	9
Age (in years)	Median [IQR]	67	67	68	66	65	66
Pre-menopausal	Yes	18	9	7	2	0	0
	No	678	315	297	56	3	7
	Unknown/Pregnant	15	5	8	0	0	2
Surgery	Complete Mastectomy	428	187	196	36	3	6
	Partial Mastectomy	274	139	111	21	0	3
	Other	9	3	5	1	0	0
Axillary Node Dissection	Yes	675	308	298	57	3	9
	No	36	21	14	1	0	0
Breast/chest wall radiation therapy	Yes	372	180	153	34	0	5
	No	339	149	159	24	3	4
Adjuvant Tamoxifen	Yes	711	329	312	58	3	9
	No	0	0	0	0	0	0
Adjuvant Chemo-therapy	Yes	0	0	0	0	0	0
	No	711	329	312	58	3	9
Tumor Size (cm)	Median [IQR]	2.2	2.0	2.5	2.5	2.5	3.0
T Stage (Clinical)	T0/IS	0	0	0	0	0	0
	T1	298	155	113	24	3	3
	T2	346	147	169	27	0	3
	T3	18	10	5	3	0	0
	T4	28	9	15	1	0	3
	TX	21	8	10	3	0	0
#Positive Nodes	0	199	83	91	18	0	7
	1-3	328	162	139	24	1	2
	4-9	111	49	51	10	1	0
	10+	26	8	16	2	0	0
	Unknown	47	27	15	4	1	0
Grade	Grade 1: well differentiated	24	20	2	1	0	1
	Grade 2: moderately differentiated	306	169	119	13	0	5
	Grade 3: poorly differentiated	338	117	173	43	2	3
	Unknown	43	23	18	1	1	0
histologic subtype	ductal NOS	642	289	288	54	3	8
	lobular	54	30	19	4	0	1
	mucinous	7	4	3	0	0	0
	tubular	5	5	0	0	0	0
	medullary	2	1	1	0	0	0
	apocrine	1	0	1	0	0	0
Lymphovascular invasion	Yes	444	184	215	39	1	5
	No	230	122	84	18	2	4
	Unknown	37	23	13	1	0	0
Clinical estrogen receptor status (DCC)	missing	6	4	2	0	0	0
	negative (0-9 fmol/mg)	9	3	2	4	0	0
	Positive (>10 fmol/mg)	696	322	308	54	3	9
Immunohisto-chemical ER	negative	0	0	0	0	0	0
	positive	711	329	312	58	3	9

[0161] Based on the nearest PAM50 centroid, a total of 329 (46.3%) of these clinically ER positive cases were assigned as Luminal A, 312 (43.8%) as Luminal B, 58 (8.2%) as HER2-enriched, 3 (0.4%) as Basal-like, and 9 (1.3%) as Normal-like intrinsic breast cancer subytpes by gene expression (Table 13). For the nine cases assigned as Normal-like, the histology was reviewed, using the tissue microarray cores taken from the same area of the source block. In eight of these nine cases, viable invasive cancer cells were absent or rare in an immediately adjacent core, consistent with the normal-like expression profile representing an inadequate tumor sampling. Normal-like cases were therefore excluded from further analysis.

[0162] Intrinsic biological subtype was strongly prognostic by Kaplan-Meier analysis (FIGS. **4**A and **4**B). In the British Columbia population at the time of sample acquisition for this study, many patients with a clinically low risk profile received no adjuvant systemic therapy[Olivotto 2005]. In contrast, those receiving adjuvant tamoxifen who are the subjects in this study comprised a higher clinical risk group, with overall 10 year distant relapse-free survival rates of 62% and breast cancer disease-specific survival rates of 72%. Those determined by the PAM50 assay to have a Luminal A profile had a significantly better outcome (10 year relapse free survival 74%, disease-specific survival=83%) than Luminal B, HER2-enriched or basal like tumors.

[0163] All cases in this study were positive for estrogen receptor by centrally-assessed immunohistochemistry [Cheang 2006], and 98.7% were also positive by clinical dextran-charcoal coated biochemical assay. Despite this, the PAM50 qPCR panel assigned 10% of cases to non-luminal subtypes, mostly HER2-enriched, as was previously observed when interrogating published datasets for expression of the PAM50 genes (Example 2).

[0164] For this cohort of clinically estrogen receptor positive women, uniformly treated with tamoxifen as their sole adjuvant systemic therapy, a multivariable Cox model was constructed to test the independent value of PAM50 subtype against patient age and the standard clinicopathologic factors of tumor size, nodal status, histologic grade and HER2 expression (Table 16). Intrinsic biological subtype remained significant in the multivariable model, as were nodal status and tumor size, but grade and clinical HER2 status, significant in univariate analysis in this cohort, did not contribute significant independent prognostic information for either relapse-free or disease-specific survival in the multivariate model incorporating the PAM50 result.

TABLE 16

Cox model univariate and multivariate analyses incorporating PAM50 biological subtype for relapse-free and breast cancer disease-specific survival among (A) 604 women with ER positive, tamoxifen-treated breast cancer with complete data for all covariates for relapse-free survival, and (B) breast cancer disease-specific survival (BCDSS; excludes 2 cases with unknown cause of death).

Clinical	univariate relapse-free survival		multivariate relapse-free survival	
endpoint	hazard ratio (95% CI)	p-value	hazard ratio (95% CI)	p-value
age (continuous)	1.00 (0.990-1.02)	0.53	0.996 (0.981-1.01)	0.62
grade (1 or 2) vs. 3	1.45 (1.12-1.89)	0.0047	1.11 (0.846-1.46)	0.45
percent nodes positive				
0 vs. (>0 to <25%)	1.66 (1.15-2.39)	0.0070	1.76 (1.22-2.55)	0.0028
0 vs. ≥25%	2.98 (2.10-4.22)	7.3E−10	2.85 (2.00-4.06)	6.3E−9
tumor size ≤2 cm vs. >2 cm	2.02 (1.55-2.65)	2.5E−7	1.71 (1.30-2.24)	1.3E−4
HER2 (IHC) {0, 1 or 2+ FISH negative} vs. {2+ FISH positive, or 3+}	1.52 (1.04-2.23)	0.032	1.24 (0.813-1.88)	0.32
PAM50 subtype				
Luminal A vs. Luminal B	1.73 (1.31-2.28)	1.0E−4	1.62 (1.22-2.16)	9.2E−4
Luminal A vs. Her2-Enriched	1.86 (1.18-2.92)	0.0074	1.53 (0.929-2.52)	0.095
Luminal A vs. Basal-like	76.4 (9.79-597)	3.5E−5	62.5 (7.87-496)	9.2E−5

TABLE 16-continued

Cox model univariate and multivariate analyses incorporating PAM50 biological subtype for relapse-free and breast cancer disease-specific survival among (A) 604 women with ER positive, tamoxifen-treated breast cancer with complete data for all covariates for relapse-free survival, and (B) breast cancer disease-specific survival (BCDSS; excludes 2 cases with unknown cause of death).

B.

Clinical endpoint	Univariate BCDSS hazard ratio (95% CI)	Univariate BCDSS p-value	Multivariate BCDSS hazard ratio (95% CI)	Multivariate BCDSS p-value
age (continuous)	1.02 (0.999-1.03)	0.069	1.01 (0.988-1.02)	0.56
grade (1 or 2) vs. 3	1.43 (1.07-1.91)	0.015	1.05 (0.988-1.02)	0.76
percent nodes positive				
0 vs. (>0 to <25%)	1.56 (1.03-2.37)	0.034	1.68 (1.11-2.56)	0.015
0 vs. ≥25%	3.22 (2.19-4.73)	2.4E−9	3.04 (2.06-4.48)	2.3E−8
tumor size ≤2 cm vs. >2 cm	2.29 (1.96-3.10)	8.0E−8	1.90 (1.40-2.58)	4.3E−8
HER2 (IHC) {0, 1 or 2+ FISH negative} vs. {2+ FISH positive, or 3+}	1.54 (1.01-2.35)	0.043	1.19 (0.755-1.86)	0.46
PAM50 subtype				
Luminal A vs. Luminal B	2.05 (1.50-2.80)	6.0E−6	1.90 (1.37-2.62)	1.0E−4
Luminal A vs. Her2-Enriched	2.2 (1.33-3.64)	0.0021	1.85 (1.07-3.20)	0.028
Luminal A vs. Basal-like	104 (13.1-832)	1.2E−5	91.1 (11.2-743)	2.5E−5

[0165] A risk-of-relapse (ROR) score can be calculated from the PAM50 qPCR panel. Both the ROR-S(based only on molecular subtyping from the PAM50 panel) and ROR-C (combining subtype and tumor size information) scores are highly prognostic in a population homogeneously treated with adjuvant tamoxifen, to a series containing large numbers of node positive cases, and to the endpoint of breast cancer-specific survival (FIGS. **5**A and **5**B).

[0166] As shown in FIGS. **6**A and **6**B, the ROR-C algorithm is not only highly prognostic among node negative patients, but reveals even wider differences in disease-specific survival among node positive patients. The algorithm identifies 16% of clinically ER positive patients (treated with adjuvant tamoxifen but not chemotherapy) who, despite being node positive, are classed as low risk, and these women have a 10 year disease-specific survival rate of 89%.

[0167] As a continuous variable, ROR-C has a significant interaction with percentage of positive lymph nodes, and borderline significant interaction with nodal stage (Table 17). Nodal stage is a significant predictor among patients with moderate to high ROR-C values (>23.5), but among patients with low ROR-C scores, outcomes are good regardless of nodal status (FIGS. **7**A to **7**D and FIG. **8**).

[0168] TABLE 17 Interaction test between PAM50- and tumor size-derived ROR-C score, expressed as a continuous variable, and axillary lymph node status (A) expressed as % positive nodes or (B) categorized by nodal stage (where referrent group is node negative, N cat2=1-3 involved axillary nodes, and N cat3=4 or more involved axillary nodes). The model in Table 17A uses the proportion of positive nodes and the interaction is significant. The model in Table 17B uses 3 level node status (N-, 1-3 pos, >3 pos) and interaction is borderline.

TABLE 17A

Variable	Only main effects Hazard	Only main effects p-value	Interaction Hazard	Interaction p-value
ROR-C	1.75	1.60E−11	1.73	8.8E−11
Pos Node %	1.56	2.50E−10	1.43	0.000017
Interaction			1.17	0.043
Full vs red				0.04

TABLE 17B

Variable	Only main effects Hazard	Only main effects p-value	Interaction Hazard	Interaction p-value
ROR-C	1.77	6.20E−11	1.52	0.018
N cat2	1.8	9.40E−03	1.73	0.022
N cat3	3.88	1.20E−08	3.15	1.40E−05
ROR*N cat2			1.08	0.71

TABLE 17B-continued

	Only main effects		Interaction	
Variable	Hazard	p-value	Hazard	p-value
ROR*N cat3			1.62	0.061
Full vs red				0.11

[0169] As ROR-C includes tumor size information, to assess if the ROR algorithm gives independent additional prognostic information beyond standard clinical parameters (including tumor size) in this patient population, Cox models incorporating ROR-S were tested (Table 18). Regardless of whether the endpoint is relapse-free or disease-specific survival, or if ROR-S is included as a categorical or as a continuous variable, it remains significant, whereas grade and clinical HER2 status are not significant in multivariate analyses that include the qPCR-derived information.

TABLE 18

Cox model multivariate analysis incorporating ROR-S score for breast cancer disease-specific survival among women with ER positive, tamoxifen-treated breast cancer and complete data for all covariates. (A) ROR-S-defined risk categories, using prespecified cutpoints. (B) ROR-S as a continuous variable.

Clinical	relapse-free survival (N = 613)		disease-specific survival (N = 611)	
endpoint	hazard ratio (95% C.I.)	p-value	hazard ratio (95% C.I.)	p-value
A.				
age (continuous)	0.995 (0.980-1.01)	0.56	1.00 (0.988-1.02)	0.56
grade (1 or 2) vs. 3	1.03 (0.785-1.36)	0.81	1.00 (0.738-1.36)	1.0
percent nodes positive				
0 vs. (>0 to <25%)	1.79 (1.24-2.58)	0.0016	1.74 (1.16-2.63)	0.0081
0 vs. ≥25%	2.87 (2.02-4.08)	4.4E−9	3.10 (2.10-4.57)	1.3E−8
tumor size ≤2 cm vs. >2 cm	1.70 (1.30-2.23)	1.2E−4	1.92 (1.42-2.61)	2.8E−5
HER2 (IHC) {0, 1 or 2+ FISH negative} vs. {2+ FISH positive, or 3+}	1.14 (0.760-1.72)	0.52	1.10 (0.701-1.74)	0.67
ROR-S (categorized)				
low vs. medium	2.00 (1.39-2.87)	1.9E−4	2.21 (1.45-3.36)	2.1E−4
low vs. high	2.68 (1.63-4.41)	1.0E−4	3.25 (1.86-5.67)	3.4E−5
B.				
age (continuous)	0.997 (0.982-1.01)	0.71	1.01 (0.989-1.02)	0.48
grade (1 or 2) vs. 3	1.06 (0.808-1.40)	0.66	1.02 (0.749-1.38)	0.92
percent nodes positive				
0 vs. (>0 to <25%)	1.77 (1.23-2.53)	0.0021	1.71 (1.13-2.58)	0.011
0 vs. ≥25%	2.87 (2.02-4.06)	3.4E−9	3.12 (2.12-4.59)	8.5E−9
tumor size ≤2 cm vs. >2 cm	1.70 (1.30-2.23)	1.2E−4	1.92 (1.41-2.60)	3.0E−5
HER2 (IHC) {0, 1 or 2+ FISH negative} vs. {2+ FISH positive, or 3+}	1.05 (0.699-1.59)	0.80	0.986 (0.628-1.55)	0.95

TABLE 18-continued

Cox model multivariate analysis incorporating ROR-S score for breast cancer disease-specific survival among women with ER positive, tamoxifen-treated breast cancer and complete data for all covariates. (A) ROR-S-defined risk categories, using prespecified cutpoints. (B) ROR-S as a continuous variable.

Clinical	relapse-free survival (N = 613)		disease-specific survival (N = 611)	
endpoint	hazard ratio (95% C.I.)	p-value	hazard ratio (95% C.I.)	p-value
ROR-S (continuous)	1.02 (1.01-1.03)	7.3E−5	1.02 (1.01-1.03)	1.0E−5

[0170] The cases in this series have previously been assessed by immunohistochemistry for ER, PR, HER2, cytokeratin 5/6, epidermal growth factor receptor, and Ki67 [Cheang 2008] [Cheang 2009], allowing intrinsic subtyping to be assigned by a surrogate immunohistochemical definition. As all cases in this series are ER positive by immunohistochemistry, all were assigned as either Luminal A (if HER2 negative and Ki67 low) or Luminal B (if HER2 positive or Ki67 high). The availability of qPCR subtyping assignments allows a comparison with immunohistochemical assignment on the same material, against patient outcome in this homogeneously-treated cohort. A total of 606 cases had sufficiently complete immunohistochemical and qPCR data for assignment to a Luminal subtype by both methods. Among these, 255 were assigned as Luminal A and 193 as Luminal B by both methods, whereas 99 were assigned Luminal A by immunostain but Luminal B by qPCR, and 59 as Luminal B by immunostain but Luminal A by qPCR, for a concordance of 74%, kappa=0.48. Where the results were discordant, only the cases assigned as Luminal B by PCR had significantly poorer outcome than those concordantly assigned as Luminal A. In multivariable analysis among these cases, both immunohistochemical and PAM50 assignment are independently significant predictors for relapse-free survival, whereas grade and HER2 status fall out of the model (Table 19). For disease-specific survival, PAM50 is significant whereas immunohistochemistry is borderline. The magnitude of the identified hazard is higher with the qPCR assignment for both endpoints. In a step-wise Cox regression model incorporating both immunohistochemical and qPCR assignment, only qPCR stays significant.

TABLE 19

Cox model multivariate analyses for Luminal cases, comparing the prognostic information from intrinsic subtyping by immunohistochemistry versus PAM50 qPCR.

Clinical endpoint	immunohistochemical subtype hazard ratio (95% CI)	p-value	PAM50 qPCR subtype hazard ratio (95% CI)	p-value
A. Relapse-free survival (N = 606)				
age (continuous)	0.992 (0.98-1.01)	0.36	0.990 (0.97-1.01)	0.26
grade (1 or 2) vs. 3	1.18 (0.89-1.57)	0.24	1.12 (0.84-1.49)	0.43
percent positive nodes				
0 vs. (>0 to <25%	1.66 (1.11-2.48)	0.014	1.68 (1.12-2.50)	0.012
0 vs. ≥25%	2.86 (1.95-4.19)	7.2E−8	2.93 (2.00-4.30)	3.8E−8
tumor size ≤2 cm vs. >2 cm	1.80 (1.34-2.42)	8.6E−5	1.81 (1.35-2.42)	7.4E−5
HER2 (IHC) {0, 1, or 2+ FISH negative} vs. {2+ FISH positive or 3+}	1.21 (0.74-1.99)	0.45	1.30 (0.81-2.09)	0.27
Luminal B vs. Luminal A	1.38 (1.02-1.86)	0.035	1.61 (1.20-2.16)	0.0014
B. Breast cancer disease-specific survival (N = 605; excludes one death of uncertain cause)				
age (continuous)	1.00 (0.98-1.02)	0.67	1.00 (0.98-1.02)	0.89
grade (1 or 2) vs. 3	1.14 (0.83-1.55)	0.42	1.05 (0.77-1.44)	0.74
percent positive nodes				
0 vs. (>0 to <25%	1.44 (0.92-2.26)	0.106	1.50 (0.96-2.34)	0.077
0 vs. ≥25%	2.79 (1.84-4.23)	1.2E−6	2.88 (1.90-4.38)	5.8E−7
tumor size ≤2 cm vs. >2 cm	2.07 (1.48-2.89)	1.8E−5	2.06 (1.48-2.87)	1.7E−5

TABLE 19-continued

Cox model multivariate analyses for Luminal cases, comparing the prognostic information from intrinsic subtyping by immunohistochemistry versus PAM50 qPCR.

Clinical endpoint	immunohistochemical subtype hazard ratio (95% CI)	immunohistochemical subtype p-value	PAM50 qPCR subtype hazard ratio (95% CI)	PAM50 qPCR subtype p-value
HER2 (IHC) {0, 1, or 2+ FISH negative} vs. {2+ FISH positive or 3+}	1.27 (0.75-2.15)	0.38	1.29 (0.78-2.13)	0.32
Luminal B vs. Luminal A	1.38 (0.99-1.93)	0.060	1.89 (1.36-2.62)	1.5E−4

Results from Adjuvant! Predictions

[0171] A comparison of the outcome predicted by the Adjuvant! model with outcome predicted by the ROR model was made in a cohort of breast cancer patients. This cohort consists of 806 patients diagnosed with invasive, estrogen receptor positive breast cancer, between the dates of 1986 and 1992. All patients had primary surgery and adjuvant systemic therapy with tamoxifen alone; none of these patients were treated with chemotherapy. The Adjuvant prognostic model was used to calculate the probability of breast cancer specific survival (BCSS) at 10 years using the standard clinicopathological features of patient age, tumor size, histological grade, lymphovascular invasion, and number of positive lymph nodes. All patients were ER positive, and the risk of breast cancer death was adjusted for adjuvant tamoxifen therapy.

[0172] Of the 806 patients, 748 had sufficient clinicopathological data to obtain an Adjuvant estimate of BCSS. The remaining 58 patients had either missing tumor size or missing lymph node data. The mean Adjuvant predicted BCSS was 73.7%. This corresponds to the observed BCSS of 73.2%. The cohort was then divided into subgroups based on the Adjuvant predicted BCSS at 10 years (Table 20).

TABLE 20

Risk Category	Adjuvant! Predicted 10-year BCSS	N	Number of Events
1	90-100%	122	16
2	80-90%	164	32
3	70-80%	168	60
4	<70%	292	121

[0173] The observed BCSS at 10 years, for each of the Adjuvant risk groups was similar to the BCSS predicted by Adjuvant (Table 21). One notable exception is the lowest risk group, in subgroup with an Adjuvant! predicted BCSS of 90-100%, the observed BCSS at 10 years is 89%. Consequently, it appears that Adjuvant is overestimating survival is this low risk group. This is consistent with the validation study of Adjuvant! using the BCOU database (Olivotto et al. (2005)). In this validation study, it was found that Adjuvant underestimated breast cancer deaths by 4.9% in the subgroup with T1N0 disease. In the subgroup of patients with a predicted BCSS of 90-100%, 87 of 122 patients had T1N0 breast cancer.

TABLE 21

Adjuvant Risk Group	Mean Adjuvant Predicted BCSS at 10 years	Observed BCSS at 10 years
90-100%	94%	89%
80-90%	85%	83%
70-80%	76%	75%
<70%	58%	61%

[0174] ROR-S using qRT-PCR data from 50 genes was then used to separate each Adjuvant! group into low vs. medium/high risk (Table 22). Due to the relatively small size of each group, medium and high risk groups were combined to improve statistical power. Also, because the Adjuvant! risk subgroups are already defined using clinical factors, ROR-S (rather than ROR-C) was applied.

TABLE 22

Adjuvant Risk Group	ROR-S Low	ROR-S Medium or High
90-100%	47	75
80-90%	56	108
70-80%	43	125
<70%	58	234

[0175] Kaplan-Meier analysis was then performed separately on each Adjuvant risk group, and differences in survival between the low vs. med/high risk ROR-S groups were tested using the log-rank test (Table 23). It was observed that ROR-S could isolate a low risk subgroup in each of the Adjuvant Risk Groups. Statistically significant differences in BCSS were found for low risk vs. medium/high risk patients, in all subgroups except for the 90-100% group.

TABLE 23

Adjuvant Risk Group	Observed BCSS	BCSS for Low Risk ROR-S	BCSS for Med/High Risk ROR-S	Log-rank Test of ROR-S
90-100%	89%	93%	85%	p = 0.058
80-90%	83%	92%	78%	p = 0.020
70-80%	75%	95%	68%	p = 0.005
<70%	61%	71%	58%	P = 0.009

[0176] In this low risk group, ROR-S is not quite statistically significant (p=0.058). However, this group does provide some convincing evidence that the ROR-S is adding additional prognostic information to Adjuvant!. A Kaplan-Meier analysis of the same group but with Adjuvant! predicted BCSS 90-95% vs. 95-100% is shown in FIG. **9**.

[0177] In the intermediate risk groups, ROR-S performs well in identifying low risk vs. higher risk patients. In both the 80-90% group (FIG. **10**A) and 70-80% group (FIG. **10**B), the ROR-S identifies subgroups with 10 year BCSS >90%. This is an important result as ROR-S identifies traditionally high-risk patients that do well without chemotherapy.

[0178] In the very high risk subgroup identified by Adjuvant! (Predicted BCSS <70%), ROR—S is still able to identify distinct prognostic groups (FIG. **10**C).

Discussion

[0179] Previous studies have established that intrinsic biological signatures characteristic of Luminal A, Luminal B, HER2-Enriched and Basal-like subtypes are present and have prognostic significance in breast cancer cohorts from multiple different institutions, profiled with several gene expression microarray platforms [Calza 2006] [Kapp 2006] [Hu 2006][Fan 2006]. In order to identify these subtypes on standard formalin-fixed, paraffin-embedded pathology specimens, a quantitative reverse-transcriptase PCR test [Mullins 2007] was developed that identifies these subtypes based on a panel of 50 genes.

[0180] The analysis reported here consists exclusively of qPCR-based testing, applied to a series of relatively old-age (15-20 years) paraffin blocks with long and detailed follow-up, allowing analysis not only of relapse-free survival, but also of breast cancer disease-specific survival. The present study consists of women with estrogen receptor positive breast cancer who received hormonal therapy (tamoxifen) as their sole adjuvant treatment, a group of particular clinical importance and contemporary relevance. Estrogen receptor and HER2 status were centrally determined. 70% of these women were node positive at presentation, and in current practice would usually be recommended to receive adjuvant chemotherapy. The PAM50 subtype assignment as determined by PCR is highly prognostic in these women. Subtype remains significant in multivariate analysis, whereas grade and clinical HER2 status do not. Findings using the commonly-employed surrogate endpoint of relapse-free survival all hold for breast cancer disease-specific survival.

[0181] Although the patients from this cohort were treated more than 20 years ago, the findings from this study remain relevant to the treatment of breast cancer patients with a moderate risk of relapse. Such patients may derive significant benefit from adjuvant hormonal therapy but the further addition of chemotherapy may have modest effects (2-5% improvement in 10-year relapse free survival). While the decision to pursue adjuvant chemotherapy is an individual decision made by the patient and consulting oncologist, improved prognostication will facilitate therapeutic decision making.

[0182] A Risk of Relapse score was developed and validated on microarray data from node negative patients who received no adjuvant systemic therapy (Example 2), against the endpoint of relapse-free survival. This algorithm is shown to predict pathologic complete response in a published neoadjuvant T/FAC clinical trial dataset of 133 patients, and, in its qPCR format, to predict relapse-free survival in a cohort of 279 heterogeneously-treated women with breast cancer. ROR scores generated by qPCR from paraffin block specimens are also prognostic in tamoxifen-treated, estrogen positive women, in both node-negative and node-positive subsets. ROR-C identifies a group of low risk patients among whom even nodal status is not a predictor, and who might therefore not require treatment approaches usually reserved for node positive patients including, for example, third generation chemotherapy regimens and chest wall radiation.

[0183] Very few cases (1.3%) are classified as Normal-like using the PCR assay, as compared to 12% when the PAM50 classifier is applied to DNA microarray data from large sets of primary breast cancers. DNA microarray analyses utilize homogenized tumor specimens that, despite gross dissection to enrich for tumor, may still contain significant amounts of normal breast tissue. In contrast, the PAM50 qPCR assay is performed on a pathologist-guided tissue core, based on direct microscopic identification of a representative area of pure tumor in the source block. This difference likely accounts for the much lower frequency of Normal-like profiles obtained using the PAM50 qPCR method applied to paraffin blocks. Review of the histology, as represented on tissue microarray cores extracted from the immediately adjacent tissue, is consistent with inadequate tumor representation being responsible for a normal-like profile in eight of the nine normal-like cases.

[0184] As was previously noted based on interrogation of published datasets with the PAM50 classifier, this assay identifies ER-negative biological subtypes among clinically ER positive women even in a setting where the tumor is positive by both immunohistochemical and ligand-binding assays. Fully 10% of cases are re-assigned to non-luminal subtypes, and these tamoxifen-treated women had poor outcomes, compatible with a biological reality of hormone independence. Clinical measurements of ER and HER2 status, on their own, can stratify breast cancer patients into prognostic and predictive subgroups[Hayes 2007]. Nevertheless, relying on measurements of single genes (ER, PR) to assign breast cancer prognosis and treatment risks not only the problems of false positive and negative single measurements, but also the possibility that a tumor's underlying biology may be hormone independent (despite one member of the pathway being expressed at the protein level). In this respect, the information provided by concurrently measuring 50 genes, including others in the estrogen response pathway together with positive markers of other biological subtypes, is likely to be a more accurate reflection of the underlying tumor biology [Oh 2006].

[0185] Larger immunohistochemical surrogate panels have been linked to expression profile gold standards and can provide more information than simple measurement of ER, PR and HER2 [Cheang 2008b][Cheang 2009]. Limited antibody panels are easily applied to standard paraffin blocks, and can add significant prognostic information beyond standard clinicopathologic risk factors [Ross 2008]. In this study, a direct comparison of an established six immunostain panel (ER, PR, HER2, Ki67, cytokeratin 5/6 and epidermal growth factor receptor) against the 50 gene qPCR assay, was made using the same source blocks. Each method adds significant prognostic information beyond standard factors. However, in this set of clinically ER positive patients, there were many discrepant assignments to an intrinsic biological subtype, and the qPCR approach was better at predicting outcome in these cases.

[0186] In multivariate analysis incorporating the main clinical risk factors, grade is no longer significant when PAM50 subtype or ROR is included. In comparison with other signatures such as the recurrence score and genomic grade indexes [Paik 2004] [Ivshina 2006] [Sotiriou 2006], the PAM50 also has the advantage of discriminating high risk cases into Luminal B, HER2-Enriched and Basal-like subtypes, who are likely to respond differently to systemic therapy options (for example, hormonal, anti-HER2, and anthracycline vs. non-anthracycline chemotherapy regimens). The assay is also easier to perform, as it does not require frozen tissue [Glas 2006] nor manual microdissection of cut sections [Paik 2004] and can be readily applied to standard paraffin blocks including archival tissues such as those from clinical trials. However, the assay can be performed on these types of samples if desired. Because the PAM50 assay was designed to reflect the major features of the underlying biology of breast cancer, as opposed to being optimized against outcome in a particular population, it is particularly likely to extrapolate well onto other patient cohorts, and remain predictive [Rouzier 2005]. In this study, it was demonstrated for the first time that the PAM50 qPCR assay has significant and independent prognostic capacity among estrogen receptor positive, tamoxifen treated women, whether node positive or node negative. The assay identifies up to 10% cases that were clinically determined to be ER positive (by immunohistochemistry and ligand-binding assay) as falling into ER negative high-risk groups, replaces grade and HER2 status in multivariate prognostic models, and is superior to immunohistochemical subtyping and clinical risk classifiers.

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[0214] All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[0215] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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tgccgcagaa ctcacttg 18

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ccatcaacat tctctttatg aacg 24

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atcaactccc aaacggtcac 20

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gacttcaggg tgctggac 18

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cagacatgtt ggtattgcac att 23

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gatggtagag ttccagtgat t 21

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catttgccgt ccttcatcg 19

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gcaggtcaaa actctcaaag 20

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<210> SEQ ID NO 100
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What is claimed is:

1. A method of classifying a breast cancer intrinsic subtype in a test sample comprising:

a) detecting the RNA expression level of at least 40 of the intrinsic genes listed in Table 1 in a plurality of training breast cancer samples to generate a first gene expression profile, wherein each of the Luminal A (LumA), Luminal B (LumB), Basal-like (Basal), and HER2-enriched (HER2) intrinsic subtypes is represented in the plurality of training breast cancer samples;

b) constructing centroids for each of the breast cancer intrinsic subtypes in the training samples by comparing the first gene expression profile of the training samples to the gene expression data deposited as accession number GSEI0886 in the National Center for Biotechnology Information Gene Expression Omnibus utilizing a nearest centroid algorithm;

c) contacting said test sample with a composition comprising a plurality of fluorescently labeled nucleic acid probes of at least 500 nucleotides in length to form a plurality of hybridization complexes, wherein each complex within said plurality of complexes comprises at least one probe in the plurality of probes and at least one RNA transcript for at least one of at least 40 intrinsic genes listed in Table 1;

d) detecting the fluorescently labeled nucleic acid probes in the complexes formed in step (c) to determine the RNA expression level of the at least 40 intrinsic genes;

e) generating a second gene expression profile based on said expression of said intrinsic genes in the test sample;

f) comparing the second gene expression profile of the test sample to each of the centroids constructed in step (b) by calculating the distance of the second gene expression profile of the test sample to each of the centroids; and,

g) classifying the test sample as one of the breast cancer intrinsic subtypes having the nearest calculated distance.

2. The method of claim **1**, wherein data obtained from the gene expression profiles for the training samples and the gene expression profile for the test sample are processed via normalization methods prior to analysis.

3. The method of claim **2**, wherein said processing comprises normalization to a set of housekeeping genes.

4. The method of claim **3**, wherein said housekeeping genes are selected from MRPL19, PSMC4, SF3A1, PUM1, ACTB, GAPD, GUSB, RPLPO, and TFRC.

5. The method of claim **1**, wherein the expression profile is based on the RNA expression of at least 45 of the intrinsic genes listed in Table 1.

6. A method of classifying a breast cancer intrinsic subtype in a test sample comprising:

a) detecting the RNA expression level of at least 40 of the intrinsic genes listed in Table 1 in a plurality of training breast cancer samples to generate a first gene expression profile, wherein each of the Luminal A (LumA), Luminal B (LumB), Basal-like (Basal), and HER2-enriched (HER2) intrinsic subtypes is represented in the plurality of training breast cancer samples;

b) constructing centroids for each of the breast cancer intrinsic subtypes in the training samples utilizing a nearest centroid algorithm;

c) contacting the test sample with a composition comprising a plurality of fluorescently labeled nucleic acid probes of at least 500 nucleotides in length to form a plurality of hybridization complexes, wherein each complex within said plurality of complexes comprises at least one probe in the plurality of probes and at least one RNA transcript for at least one of at least 40 intrinsic genes listed in Table 1;

d) detecting the fluorescently labeled nucleic acid probes in the complexes formed in step (c) to determine the RNA expression level of the at least 40 intrinsic genes;

e) generating a second gene expression profile based on said expression of the intrinsic genes in the test sample;

f) comparing the second gene expression profile of the test sample to each of the centroids constructed in step (b) by calculating the distance of the second gene expression profile of the test sample to each of the centroids; and,

g) classifying the test sample as one of the breast cancer intrinsic subtypes having the nearest calculated distance.

* * * * *