Gene expression pathway analysis to predict response to neoadjuvant docetaxel and capecitabine for breast cancer

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Abstract Neoadjuvant chemotherapy has been shown to be equivalent to post-operative treatment for breast cancer, and allows for assessment of chemotherapy response. In a pilot trial of docetaxel (T) and capecitabine (X) neoadjuvant chemotherapy for Stage II/III BC, we assessed correlation between baseline gene expression and tumor response to treatment, and examined changes in gene expression associated with treatment. Patients received four cycles of TX. Tumor tissue obtained from MammotomeTM core biopsies pretreatment (BL) and post-cycle 1 (C1) of TX was flash frozen and stored at -70°C until processing. Gene expression analysis utilized Affymetrix HG-U133 Plus 2.0 Gene-Chip arrays. Statistical analysis was performed using BRB Array Tools after RMA normalization. Gene ontology (GO) pathway analysis used random variance t tests with a

significance level of $P < 0.005$. For gene categories identified by GO pathway analysis as significant, expression levels of individual genes within those pathways were compared between classes using univariate t tests; those genes with significance level of $P < 0.05$ were reported. PAM50 analyses were performed on tumor samples to investigate biologic subtype and risk of relapse (ROR). Using GO pathway analysis, 39 gene categories discriminated between responders and non-responders, most notably genes involved in microtubule assembly and regulation. When comparing pre- and post-chemotherapy specimens, we identified 71 differentially expressed gene categories, including DNA repair and cell proliferation regulation. There were 45 GO pathways in which the change in expression after one cycle of chemotherapy was significantly different among responders and non-responders. The majority of tumor samples fell into the basal-like and luminal B categories. ROR scores decreased in response to chemotherapy; this change was more evident in

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samples from patients classified as responders by clinical criteria. GO pathway analysis identified a number of gene categories pertinent to therapeutic response, and may be an informative method for identifying genes important in response to chemotherapy. Larger studies using the methods described here are necessary to fully evaluate gene expression changes in response to chemotherapy.

Keywords Neoadjuvant · Gene expression · Chemotherapy response · Microtubules · DNA repair · PAM50

Introduction

Breast cancer mortality has decreased over the last several decades, likely due to a combination of mammographic screening and improvements in systemic therapy [1]. A substantial proportion of women diagnosed with breast cancer in the United States receive adjuvant chemotherapy [2], but our ability to predict a priori which patients are most likely to benefit from chemotherapy is limited. Recent advances in molecular profiling of tumor tissue suggest that a more personalized approach to breast cancer treatment may be possible [3, 4]. Trials that utilize pre-operative or neoadjuvant chemotherapy are uniquely situated to evaluate the molecular correlates of chemosensitivity because tumor response to treatment can be assessed in real time. These studies also have the added advantage of allowing the assessment of paired pre- and post-chemotherapy tumor specimens in order to better understand the biology and genetic heterogeneity of breast cancer. This could, in turn, lead to a more individualized approach to treatment—allowing for a specific selection of the chemotherapeutic agent or agents to which a particular tumor is most susceptible.

It is likely that the genes in which expression changes in response to chemotherapy are different among those who respond to therapy (i.e., genes conferring sensitivity) and those who do not (i.e., genes that confer resistance). In this study, we sought to identify genes associated with chemotherapy sensitivity and resistance. We utilized pre- and post-treatment biopsies from a phase II study of neoadjuvant docetaxel and capecitabine chemotherapy to assess the correlation between baseline gene expression and tumor response to treatment, and to examine changes in gene expression associated with treatment. We also sought to assess the accuracy of gene expression profiles identified in other studies as predictive of response in our dataset.

Materials and methods

The design of this phase II neoadjuvant treatment study has been previously described [5]. Briefly, women with newly

diagnosed stage 2 or 3 breast cancer with a tumor size of ≥ 2 cm, normal laboratory parameters, and a Zubrod Performance status from 0 to 2 were eligible. Patients were excluded if they had a bleeding disorder, a history of cardiac disease, or if they were pregnant or lactating. The protocol was reviewed and approved by the Institutional Review Board of the National Cancer Institute.

All patients received four cycles of docetaxel and capecitabine administered every 21 days. Patients were initially treated with docetaxel (75 mg/m² i.v.) on day 1 and capecitabine (1,000 mg/m² p.o.) twice daily on days 2–15 every 21 days for four cycles. Due to excessive toxicity in the first 10 patients treated on protocol, both agents were dose reduced (docetaxel to 60 mg/m² and capecitabine to 937.5 mg/m² twice daily). After completing four cycles of docetaxel/capecitabine (TX), all patients received four cycles of adriamycin (60 mg/m²) and cyclophosphamide (600 mg/m²) on day 1 and every 21 days; six patients with a poor response to the initial chemotherapy received adriamycin and cyclophosphamide (AC) prior to surgery, the remainder had this additional therapy post-operatively. All tumor samples used in this analysis were taken prior to AC chemotherapy, and tumor measurements used to assess response were also taken prior to AC. Radiation therapy was individualized based on type of surgery, tumor size, and lymph node involvement. Patients with hormone receptor positive disease received either tamoxifen and/or an aromatase inhibitor after completing chemotherapy, surgery, and radiation treatment. No patients on this study received adjuvant or neoadjuvant trastuzumab.

We recorded tumor size using bidirectional and longest dimension measurements by physical examination at baseline, after completion of TX, and prior to surgery. We classified patients as either responders or non-responders based on change in tumor size by clinical exam and pathologic response (see Table 1). For patients treated pre-operatively with AC, response was assessed at the completion of TX. Briefly, patients with a pathological complete response (three patients), microinvasive disease at surgery (two patients), or clinical complete response after four cycles of TX (three patients) were considered as responders. All patients classified as responders received AC post-operatively. Thirteen patients were considered non-responders; 11 with a partial response (17–75% residual disease after four cycles of TX) and two with progressive disease (110–154% of baseline) after four cycles of TX. Of these, both patients with progressive disease and four patients with a partial response received additional pre-operative therapy with adriamycin and cyclophosphamide, however, the tumor measurements used to determine response category were taken prior to this additional treatment. All 21 patients are included in the baseline analysis, and 14 patients with paired baseline

Table 1 Tumor characteristics, clinical response, biologic subtype by PAM50, and risk of recurrence (ROR) score for patient tumors specimens included in analysis

Pt no.	Stage at diagnosis	Receptor status ^a	Clinical tumor measurement at baseline (cm)	Clinical tumor measurement after four cycles of TX (cm)	Pathologic findings at surgery	RECIST clinical response	Response category	Baseline PAM50	Post-chemo PAM50	Baseline ROR	Post-chemo ROR
Responders											
18 ^b	T2N1M0	ER-/PgR+/HER2-	7	0	1.5 cm	CR	R	Basal	Normal	46	-2
27 ^b	T3N1M0	ER+/PgR+/HER2+	7	1	Microfocus of IDC	PR	R	Her2	LumA	46	1
25 ^b	T3N0M0	ER+/PgR+/HER2-	5.3	1.5	1.2 cm ^c	PR	R	Lum A	LumA	29	8
12 ^b	T3N1M0	ER+/PgR+/HER2-	12	0	No residual disease	CR	R	Lum B	LumA	49	-1
14 ^b	T2N0M0	ER+/PgR-/HER2+	4.5	0	No residual disease	CR	R	Lum B	LumA	48	28
6 ^b	T2N0M0	ER+/PgR+/HER2-	3.5	0	Foci of residual disease—size not documents	CR	R	Lum B	LumA	82	8
3	T2N1M0	ER+/PgR-/HER2+	5	0.8	DCIS; few foci IDC	PR	R	Lum B	n/a	43	n/a
21	T2N0M0	ER-/PgR+/HER2-	3.5	0	1.2 cm	CR	R	Basal	n/a	48	n/a
Non-responders											
8 ^b	T3N1M0	ER-/PgR-/HER2-	8.5	3	3 cm	PR	NR	Basal	Basal	75	70
29 ^b	T3N1M0	ER-/PgR+/HER2+	7.5	3.5	3.5 cm ^c	PR	NR	Basal	Her2	69	69
20 ^b	T3N0M0	ER-/PgR-/HER2-	12	9	2.0 cm ^c	PR	NR	Basal	Basal	56	48
13 ^b	T3N1M0	ER-/PgR+/HER2-	5.5	8.5	3.5 cm ^c	PD	NR	Basal	Basal	75	20
19 ^b	T3N3M0	ER-/PgR-/HER2-	16	11	5.5 cm ^c	PR	NR	LumA	Normal	-5	40
26 ^b	T4N0M0	ER+/PgR+/HER2-	5	3.5	3.5 cm ^c	PR	NR	LumB	LumA	39	32
15 ^b	T3N1M0	ER+/PgR+/HER2+	16	5	8 cm	PR	NR	LumB	LumA	48	17
17 ^b	T3N1M0	ER-/PgR-/HER2-	8	2.2	1.2 cm	PR	NR	Normal	Basal	4	52
4	T3N0M0	ER+/PgR-/HER2-	6	1	1 cm	PR	NR	Normal	n/a	24	n/a
24	T4N0M0	ER-/PgR-/HER2-	13.6	4	2.5 cm	PR	NR	Basal	n/a	55	n/a
16	T3N1M0	ER-/PgR-/HER2-	10	11	1 cm ^c	SD	NR	Basal	n/a	72	n/a
7	T3N0M0	ER+/PgR-/HER2-	6	2.6	Multifocal tumor noted throughout the breast	PR	NR	LumA	n/a	18	n/a
28	T3N0M0	ER+/PgR+/HER2+	7.5	3.9	Scattered areas of infiltrating lobular carcinoma ^c	PR	NR	LumA	n/a	17	n/a

^a Estrogen receptor and/or progesterone receptor were considered positive if >1% of tumor cells stained positive by IHC; tumors were considered HER2+ if they were either 3+ by IHC or if the HER2:CEP17 ratio was >2.0 by FISH

^b Patient sample used in both baseline and paired pre- and post-chemotherapy analysis

^c Patient received AC chemotherapy prior to surgery

and post-cycle 1 evaluable specimens are included in the paired analyses (described in detail below).

Tumors were assessed for estrogen (ER) and progesterone receptor (PgR) positivity by immunohistochemical staining using the 6F11 antibody for ER and clone 16 for PgR (Novocastra, US). Tumors were considered positive for ER or PgR if >1% of cells were positive. Tumors were considered HER2 positive if they were either categorized by the reading pathologist as 3+ by immunohistochemistry (DAKO HercepTest) or if the HER2:CEP17 ratio by fluorescence in situ hybridization (FISH) was >2.0.

Tissue collection and processing

Tumor tissue was obtained by MammotomeTM biopsy prior to treatment, after one cycle of docetaxel/capecitabine chemotherapy, and from the definitive surgical specimen (OR). This report presents analyses using only baseline and post-cycle 1 specimens, and thus the results of the gene expression analyses are not influenced by the receipt of adriamycin/cyclophosphamide. Tissue was flash frozen with liquid nitrogen and stored at -70°C until processing. All samples included in the analysis were positive for malignant cells by hematoxylin and eosin staining on either formalin-fixed biopsy specimen (29%), touch prep (6%), or both (65%).

Total RNA was extracted from frozen tissues using TRIzol[®] Reagent (Life Technologies, Inc.) according to the standard protocol. The quantity and quality of the RNA was determined by the Agilent Technologies 2100 Bioanalyzer (Bio Sizing Software version A.02.01., Agilent Technologies) and the quantity determined with the Nanodrop[®] (Nanodrop Technologies). Samples containing high-quality total RNA with ratios between 1.8 and 2.1 were purified with the RNeasy Mini Kit (Qiagen) or the RNeasy Micro Kit (Qiagen), dependent on sample size. An “on column” digest was performed as part of this purification step using the RNase-Free DNase Set (Qiagen).

Double-stranded cDNA was synthesized from this preparation using the GeneChip[®] Expression 3'-Amplification Two-Cycle cDNA Synthesis Kit according to the protocol for Affymetrix GeneChip[®] Eukaryotic Target Preparation. The resulting double-stranded cDNA was purified using the GeneChip[®] Sample Cleanup Module (Qiagen). Synthesis of Biotin-Labeled cRNA was obtained by in vitro transcription of the purified template cDNA using the GeneChip[®] Expression 3' Amplification Reagents for IVT Labeling (Affymetrix). The cRNA target was purified by using the GeneChip[®] Sample Cleanup Module (Qiagen). A 1:10 dilution was made with 1 μl of the purified IVT reaction product and then run on the Agilent Technologies 2100 Bioanalyzer (Bio Sizing Software version A.02.01., Agilent Technologies) to determine

sample quality and the Nanodrop[®] for quantitation. The cRNA was then adjusted according to the Affymetrix protocol prior to fragmentation using the GeneChip[®] Sample Cleanup Module (Qiagen). One microliter was removed from the fragmentation reaction and checked on the Agilent Technologies 2100 Bioanalyzer (Bio Sizing Software version A.02.01., Agilent Technologies) to confirm proper fragmentation prior to hybridization.

Hybridization cocktails from each fragmentation reaction were prepared according to the Affymetrix GeneChip[®] protocol. The hybridization cocktail was applied to the Affymetrix HU-U133 Plus 2.0 GeneChip[®] Arrays, processed on the Affymetrix[®] Fluidics Station 400 then analyzed on the Agilent GeneArray Scanner with Affymetrix Microarray Suite version 5.0.0.032 software.

All assays were performed without knowledge of either class assignment (responder or non-responder) or time point of tumor sample obtainment.

Statistical analysis

Probe-level data were normalized and gene expression summaries were computed for each probe set using Robust Multichip Analysis (RMA). Statistical analyses were performed using BRB Array Tools version 3.4.1. All gene expression summary intensities (gene summaries) below 50 were thresholded to the value of 50 and genes showing variability significantly smaller than the median gene variability (variance-based screening criteria) were screened out ($P < 0.01$, based on χ^2 test as implemented in BRB Array Tools [6]).

Samples were clustered using agglomerative hierarchical clustering and using the mean centered 24,224 genes passing the variance-based screening with centered correlation as distance metric and average linkage as linkage function.

Gene Set Expression as implemented in BRB Array Tools was used to identify gene ontology (GO) groups that had more differentially expressed genes than expected by chance among [1] responders vs. non-responders and [2] baseline vs. post-cycle 1 specimens. Gene Set Expression method identifies which gene sets contain more differentially expressed genes than would be expected by chance using two statistics (LS and KS). We considered significant the GO categories for which the P value from the LS or KS permutation test was 0.005 or less. Each of the 24,224 genes passing the variance-based screening criteria could be mapped to more than one GO category. Overall, 1,666 GO categories were considered. Post-cycle 1 and baseline specimens were compared using methods for paired data.

In addition, we identified GO categories and genes in which the change in expression from baseline to post-cycle 1 was significantly different in responders vs. non-responders.

In order to perform this analysis, for each patient we calculated the log-ratios between post-cycle 1 and baseline gene summaries (thresholded as described above) and compared log-ratios between responders and non-responders; 19,165 genes passed the variance-based screening (applied to log-ratios and on this reduced set of samples) and 1,514 unique GO categories were considered.

In order to assess whether genes identified in other studies could predict response in our samples, we matched the 92 genes from the gene profile published by Chang et al. [7] to the probe sets of our array based on Locuslink ID, identifying 251 probe sets on the HG-U133 Plus 2.0 chip. A class predictor for response was developed using this set of genes on our data and its performance was assessed because the predictive model had to be partly re-developed on our data (only the gene list from previous study was kept fixed), its predictive accuracy was evaluated using leave-one-out cross-validation to avoid bias.

Finally, patient samples were also assessed using the PAM50 algorithm. For this analysis, raw data from baseline and post-cycle 1 specimens were processed using RMA normalization and each gene was centered to the training set median to account for array platform differences. Probesets were then mapped to Entrez gene names using the Affymetrix annotation file. Probesets that mapped to identical Entrez gene names were collapsed to the sample mean. The 50 genes of interest (listed in {Parker, 2009 #30}) were extracted and the Spearman's rank correlation between each sample and each subtype centroid was calculated. Each sample was assigned the class of the most highly correlated centroid. The algorithm outlined by Parker et al. [8] was followed to assign biologic subtype and risk of relapse (ROR) score.

Results

Patient and tumor characteristics

Patients were accrued to the study between January 2001 and August 2003. Thirty patients enrolled in the study; demographic and tumor characteristics for the entire study population have previously been described [5]. One patient withdrew after the first cycle of chemotherapy and was excluded from all analyses. Twenty-one patients had baseline tumor biopsies that contained malignant cells, and are included in the analysis of baseline gene expression in responders vs. non-responders (see Table 1). Of these, 14 patients had evaluable tumor samples at baseline and after one cycle of chemotherapy, and are included in subsequent analyses.

A clustering dendrogram of all tumor samples obtained is shown in Fig. 1. In general, samples from the same

Fig. 1 Clustering dendrogram of patient specimens. Samples were clustered using agglomerative hierarchical clustering and the mean centered 24,224 genes passing the variance-based screening with centered correlation as distance metric and average as linkage function. Number in “ID” column corresponds to Patient ID number; there are up to three samples per patient

patient at different time points clustered together. In addition, triple-negative tumors tended to cluster together, and hormone receptor positive tumors also clustered.

Gene ontology (GO) category analysis

We compared gene expression in baseline specimens among responders and non-responders, and in baseline vs. post-cycle 1 specimens, using GO categories, which describe gene products in terms of their associated biological processes, cellular components, and molecular functions. The analysis of baseline gene expression included baseline specimens from 21 patients: 8 responders and 13 non-responders. Analyses of changes in gene expression included paired baseline and post-cycle 1 specimens from 14 patients; of these six patients were responders and eight patients were non-responders.

When comparing responders with non-responders, 39 GO categories of 1,666 categories tested discriminated between the two groups at a significance level of $P < 0.005$. On average, we would expect to find eight GO categories to be differentially expressed if there was no difference between classes. GO categories that were significantly differentially expressed are listed in Table 2. The individual genes within these categories that showed differential expression ($P < 0.05$) are listed in Appendix 1. These included a number of genes involved in regulation of microtubule depolymerization (microtubule-associated protein-2; *MAP2*) and cell cycle arrest (microtubule-actin crosslinking factor 1; *MACF1*). Other genes of biologic interest that were differentially expressed included vascular endothelial growth factor-B (*VEGF-B*) and epidermal growth factor receptor (*EGFR*).

When comparing baseline tumor specimens with those obtained after one cycle of chemotherapy with docetaxel and capecitabine, 71 of 1,666 GO categories discriminated among classes at $P < 0.005$ (Table 3). On average, we would expect to find eight differentially expressed GO categories by chance alone. Individual genes that showed significantly different expression from these pathways are listed in Supplemental Appendix 2. Genes that were significantly differentially expressed included those involved in DNA repair [breast cancer 1 early onset; *BRCA1*, poly (ADP-ribose) polymerase family, member 2 and 3; *PARP2* and *PARP3*], and cell proliferation regulation [fibroblast growth factor receptor oncogene partner (*FGFR1OP*) and epidermal growth factor receptor (*EGFR*)]. In addition, a number of spindle-associated checkpoint genes exhibited differential expression including budding uninhibited by benzimidazoles proteins 1 and 3 (*BUB1* and *BUB3*), mitotic arrest deficient protein 2L1 (*MAD2L1*), and tubulin isoform gamma.

We looked for which genes had a significantly different change in expression from baseline to post-cycle 1 between responders and non-responders. In this analysis, we found 45 of 1,514 GO categories (Table 4) in which the change in expression after one cycle of chemotherapy discriminated

among the two classes (eight would be expected by chance alone). The individual genes within these categories that were statistically significantly different in this analysis are shown in Supplemental Appendix 3. Among the genes with greater changes in responders were two heat shock proteins (*HSPA9B* and *HSPA5*), inhibin beta-A, a number of ubiquitin-related genes, tumor necrosis factor alpha-induced protein 6 (*TNF α IP6*), and periostin (*POSTN*). Genes in which the change in expression was significantly greater in non-responders than in responders included transforming growth factor beta receptor III (*TGF β R3*), *VEGFB*, and fibroblast growth factor receptor-1 (*FGFR1*), and genes involved in reactive oxygen species metabolism such as glutathione peroxidase-3 (*GPX3*) and aldehyde oxidase-1 (*AOX1*).

Class prediction

In order to assess whether genes identified in other studies as important determinants of response to chemotherapy were predictive in our samples, we developed on our data set a class predictor for response using the genes identified by Chang et al. [7] and assessed its performance using leave-one-out cross-validation. The starting set of genes was the set of genes identified by Chang et al. [7] as significantly different between the classes of responders and non-responders in their data set. When developing our predictor we selected from among the Chang genes those that were significantly differentially expressed between classes at 0.1 significance level in our data set. In order to avoid introducing bias into the prediction accuracy estimate due to the use of our data for the classifier building process we used leave-one-out cross-validation. Specifically, the entire process of gene selection and model fitting was repeated for each leave-one-out cross-validation training set to ensure a fair representation of the prediction accuracy.

A compound covariate classifier for response had a sensitivity of 50% and a specificity of 61.5% estimated by cross-validation. Positive and negative predictive values were 44 and 67%, respectively. The cross-validated estimate of percent correct classification was 57%. Similar results were obtained using other prediction methods or further selecting the features to be included in the classifiers. Specifically, we restricted the analysis to the genes of Chang's list that showed a consistent "direction" in our data set, i.e., to the genes whose fold change between responders and non-responders was above or below 1 in both studies, regardless of the statistical significance in our data set. The predictive accuracy of the classifiers built using only concordant genes was similar to that noted above.

Table 2 GO categories significantly differentially expressed between responders and non-responders in baseline tumor samples

GO description	GO term	Number of genes	LS permutation <i>P</i> value	KS permutation <i>P</i> value
Lysosomal membrane	CC	5	1.00E–05	0.14641
Cytochrome-b5 reductase activity	MF	5	1.00E–04	0.0161
Calmodulin binding	MF	52	0.00019	0.00129
Copper ion binding	MF	17	0.00042	0.00034
Oxidoreductase activity, acting on NADH or NADPH	MF	33	0.00044	0.00444
L-amino acid transporter activity	MF	9	0.00082	0.09359
Copper ion transporter activity	MF	6	0.00146	0.00015
Amino acid transporter activity	MF	40	0.00171	0.0191
Tissue morphogenesis	BP	12	0.00211	0.0094
Amine transporter activity	MF	44	0.00234	0.01258
Protein autoprocessing/protein amino acid phosphorylation	BP	10	0.00274	0.00069
Fat-soluble vitamin metabolism	BP	5	0.00297	0.04321
Cell cycle arrest	BP	32	0.00297	0.03979
Diacylglycerol binding	MF	16	0.00344	0.01502
Glutathione biosynthesis	BP	10	0.00352	0.00481
Keratinization	BP	6	0.00374	0.08781
Phosphoinositide-mediated signaling	BP	23	0.00417	0.00126
L-amino acid transport	BP	6	0.00418	0.20218
Regulation of microtubule polymerization or depolymerization	BP	9	0.00431	0.09298
1-Acylglycerol-3-phosphate <i>O</i> -acyltransferase activity	MF	14	0.0044	0.18002
G-protein signaling coupled to IP3 second messenger (phospholipase C activating)	BP	21	0.00454	0.00332
Cell junction	CC	87	0.00482	0.03406
Epidermis morphogenesis	BP	7	0.00488	0.09918
Acylglycerol <i>O</i> -acyltransferase activity	MF	16	0.00495	0.06529
Protein-peroxisome targeting	BP	5	0.00533	0.00151
Epidermal growth factor receptor signaling pathway	BP	22	0.00622	0.0018
Neutral lipid metabolism	BP	8	0.01293	0.00068
Digestion	BP	16	0.01345	0.00182
Monoxygenase activity	MF	10	0.01545	0.00112
Calcium- and calmodulin-dependent protein kinase complex	CC	9	0.01608	0.00274
Smooth endoplasmic reticulum	CC	8	0.02046	0.00191
Heparin binding	MF	29	0.02593	0.00443
Ethanolaminephosphotransferase activity	MF	5	0.02861	0.00132
Nuclear localization sequence binding	MF	9	0.03631	0.00399
Water transporter activity	MF	5	0.0718	0.00183
Ethanol metabolism	BP	5	0.09696	0.00482
Peptide hormone secretion	BP	5	0.11154	0.00211
Nuclear chromatin	CC	9	0.23162	0.0036
Myeloid cell differentiation	BP	8	0.33765	0.00446

CC cellular component, *MF* molecular function, *BP* biological process

Biologic subtype and ROR score

Results of biologic subtype analysis and ROR scores are shown in Table 1. At baseline, 38% of the 21 tumors analyzed were classified as basal-like, 29% were luminal

B, 19% were luminal A, 9% were normal-like, and 5% were HER2-enriched. Luminal A tumors had the lowest ROR scores at baseline. The mean ROR was similar in responders and non-responders, although wider variation in baseline ROR was noted in non-responders (mean ROR in

Table 3 GO categories significantly differentially expressed after one cycle of chemotherapy compared with baseline

GO description	GO term	Number of genes in GO category	LS permutation <i>P</i> value	KS permutation <i>P</i> value
Chromosome pericentric region	CC	26	1.00E–05	1.00E–05
Kinetochores	CC	16	1.00E–05	1.00E–05
Condensed chromosome	CC	18	1.00E–05	0.00175
Spindle	CC	67	1.00E–05	0.00265
Chromosomal part	CC	33	1.00E–05	9.00E–05
Lamin binding	MF	5	1.00E–05	1.00E–05
M phase of mitotic cell cycle	BP	69	1.00E–05	1.00E–05
Mitotic cell cycle	BP	88	1.00E–05	1.00E–05
M phase	BP	79	1.00E–05	1.00E–04
Glucose catabolism	BP	80	1.00E–05	0.01684
DNA replication	BP	98	1.00E–05	1.00E–05
DNA repair	BP	92	1.00E–05	1.00E–05
Mitosis	BP	64	1.00E–05	1.00E–05
Monosaccharide catabolism	BP	83	1.00E–05	0.01815
Replisome	CC	15	9.00E–05	0.00108
DNA-dependent DNA replication	BP	44	9.00E–05	0.00924
Replication fork	CC	16	1.00E–04	0.00287
Glycolysis	BP	63	1.00E–04	0.0016
DNA replication initiation	BP	6	1.00E–04	0.00016
Condensed nuclear chromosome	CC	14	0.00013	0.01502
Rho GTPase activator activity	MF	14	0.00017	0.00562
Carbohydrate catabolism	BP	100	0.00031	0.04332
Mitochondrial lumen	CC	16	0.00031	0.03546
Alcohol catabolism	BP	86	0.00031	0.03545
Hexose catabolism	BP	82	0.00032	0.0261
Spindle organization and biogenesis	BP	11	0.00036	0.00177
Regulation of mitosis	BP	17	0.00036	0.00089
Positive regulation of cell proliferation	BP	49	0.00038	1.00E–05
Chromatin binding	MF	44	4.00E–04	0.01305
Chromosome segregation	BP	12	0.00048	0.00792
Nucleolus	CC	99	0.00065	0.05974
Regulation of cell cycle	BP	19	0.00086	0.0036
Mitochondrial matrix	CC	64	0.00091	0.01216
Response to radiation	BP	11	0.00095	0.00032
Ubiquinone metabolism	BP	6	0.00102	0.03387
Response to temperature	BP	9	0.00149	0.00069
Response to cold	BP	6	0.00154	0.00086
Biopolymer methylation	BP	22	0.00168	0.00038
Oxidoreduction coenzyme metabolism	BP	25	0.00194	0.25704
Mitotic spindle organization and biogenesis	BP	10	0.00196	0.00456
Polysome	CC	9	0.00198	1.00E–05
Positive regulation of cellular metabolism	BP	55	0.00211	0.03108
Coenzyme biosynthesis	BP	61	0.00251	0.00018
Translational initiation	BP	63	0.00272	0.00303
Mitochondrial part	CC	22	0.00303	0.20335
RNA polymerase II transcription factor activity enhancer binding	MF	7	0.00362	0.08965
Nuclear chromosome	CC	42	0.00376	0.05191

Table 3 continued

GO description	GO term	Number of genes in GO category	LS permutation <i>P</i> value	KS permutation <i>P</i> value
Regulation of glucose import	BP	7	0.00398	0.01164
Cell division	BP	80	0.0041	0.0135
Protein amino acid methylation/alkylation	BP	15	0.00461	0.00476
Microtubule cytoskeleton organization and biogenesis	BP	51	0.00479	0.0234
Cell fate commitment	BP	20	0.00632	0.00421
Localization of cell	BP	38	0.00702	0.00355
Microtubule-based movement	BP	84	0.00928	0.00048
Glutathione biosynthesis	BP	10	0.0097	0.00019
Regulation of translational initiation	BP	29	0.01562	0.00011
Phosphopyruvate hydratase complex	CC	5	0.01782	0.00395
Pericentriolar material	CC	7	0.01789	0.00261
Cytoskeleton-dependent intracellular transport	BP	90	0.01919	0.00122
Glutathione metabolism	BP	13	0.02492	6.00E-04
mRNA 3'-UTR binding	MF	5	0.03375	0.00172
Regulation of RNA metabolism	BP	13	0.03479	0.00275
Oxygen transporter activity	MF	7	0.0504	0.00431
Protein polymerization	BP	46	0.05402	0.0012
Cofactor transporter activity	MF	7	0.05827	0.00454
Stress-activated protein kinase signaling pathway/JNK cascade	BP	16	0.0978	0.00475
Mesoderm development	BP	14	0.10402	0.00199
Cell development	BP	24	0.13061	0.00421
Cofactor transport	BP	7	0.1456	0.00454
Ras GTPase activator activity	MF	10	0.17116	0.00448
Insoluble fraction	CC	13	0.22703	0.00299

responders = 47, interquartile range = 3; mean ROR in non-responders = 48, interquartile range = 51). In the paired analysis, tumors from patients classified as responders based on clinical criteria tended to have large decreases in ROR score from baseline to post-cycle 1 [mean post-cycle 1 ROR = 4.5 (interquartile range = 8.5) in the six patients included in the paired analysis]; conversely, smaller decreases or increases in ROR score were seen among non-responders [mean post-cycle 1 ROR = 44 (interquartile range = 27.25) among eight non-responders in the paired analysis]. Changes in ROR for each individual patient are shown in Fig. 2. Interestingly, for 11/14 patients in the paired analysis, biologic subtype changed after one cycle of chemotherapy; the most common change was from luminal B to luminal A subtype. In responders, all post-chemotherapy specimens mapped to either the luminal A or normal-like group. Among non-responders, there was greater variation in subtype after one cycle of chemotherapy. Heatmaps showing clustering of all patient samples (panel A) and baseline samples only (panel B) are shown in Fig. 3.

Discussion

In this study of gene expression by cDNA microarray analysis among breast cancer patients treated with neoadjuvant docetaxel and capecitabine, we identified a number of biologic pathways that appeared to be important in characterizing response to chemotherapy, including spindle regulation and microtubule depolymerization, DNA repair, and cellular proliferation. In addition, we found that previously published gene sets related to treatment response had modest predictive accuracy for our samples.

Docetaxel is a taxane used to treat breast cancer in both the metastatic and adjuvant settings. Taxanes bind to tubulin and inhibit microtubule depolymerization, disrupting mitotic cell division and thus leading to cell death [9]. Microtubule-associated proteins, MAP2, MAP4, and MAP-tau stabilize microtubules by binding to tubulin, and increased microtubule dynamics associated with altered MAP expression has been implicated in taxane resistance [10, 11]. In our study, non-responders had significantly higher expression of *MAP2* than responders, suggesting

Table 4 GO categories in which the change in gene expression from baseline to post-cycle 1 of chemotherapy was significantly different between responders and non-responders

GO description	GO term	Number of genes	LS permutation <i>P</i> value	KS permutation <i>P</i> value
Endoplasmic reticulum lumen	CC	19	1.00E–05	0.02961
Pattern binding	MF	45	1.00E–05	1.00E–05
Electron transporter activity	MF	73	1.00E–05	0.00539
Glycosaminoglycan binding	MF	44	1.00E–05	1.00E–05
Small protein activating enzyme activity	MF	41	1.00E–05	0.0392
Ubiquitin-like activating enzyme activity	MF	40	1.00E–05	0.02938
Polysaccharide binding	MF	44	1.00E–05	1.00E–05
Signal sequence binding	MF	16	1.00E–04	0.00115
Regulation of actin filament polymerization	BP	6	0.00018	0.00195
Actin filament polymerization	BP	12	0.00036	0.007
Response to temperature	BP	8	0.00038	0.00889
Protein translocase activity	MF	14	0.00063	0.02231
Protein-mitochondrial targeting	BP	5	9.00E–04	0.08933
Oxidoreductase activity acting on single donors with incorporation of molecular oxygen	MF	5	0.00195	0.0092
Protein polymerization	BP	32	0.00195	0.00354
Protein carrier activity	MF	17	0.00196	0.03679
Cell surface	CC	39	0.00204	0.03331
Neutral amino acid transporter activity	MF	6	0.00207	0.01723
Sodium: dicarboxylate symporter activity	MF	8	0.00216	0.02185
Cell cycle arrest	BP	20	0.00421	0.00081
Heparin binding	MF	25	0.00428	1.00E–05
Protein import	BP	32	0.00441	0.26526
Cofactor transporter activity	MF	6	0.00446	0.00871
Protein targeting	BP	65	0.00471	0.19948
Regulation of caspase activity	BP	12	0.00484	0.0657
tRNA modification	BP	13	0.00507	0.00369
RNA modification	BP	18	0.00514	0.00182
Circulation	BP	34	0.0057	0.0038
Oxygen and reactive oxygen species metabolism	BP	38	0.00881	0.00133
Oxidoreductase activity acting on CH–OH group of donors	MF	35	0.00925	0.00241
Protein tyrosine phosphatase activity	MF	13	0.00958	0.00064
STAT protein nuclear translocation	BP	7	0.01029	0.00432
tRNA metabolism	BP	47	0.01882	0.00227
Phosphoprotein phosphatase activity	MF	29	0.01954	0.00143
Muscle myosin	CC	10	0.02072	0.0026
Myosin II	CC	10	0.02072	0.0026
Fibroblast growth factor receptor activity	MF	5	0.02293	0.00435
Protein kinase A binding	MF	11	0.03001	0.00386
COPI vesicle coat	CC	7	0.03033	0.00301
FACIT collagen	CC	8	0.03366	1.00E–04
Neuromuscular junction development	BP	9	0.03667	0.00087
Anchoring collagen	CC	9	0.04456	0.00216
G-protein-coupled receptor binding	MF	31	0.0484	0.0033
Regulation of heart contraction rate	BP	10	0.05104	0.00422
Skeletal muscle fiber development	BP	10	0.05699	0.00422

Fig. 2 Change in ROR score after chemotherapy among responders (panel a) and non-responders (panel b)

that increased MAP2 protein may be involved in resistance in our patients. *MAP4* was also among the genes in the GO pathways that showed a trend toward differential expression in our analysis, but the fold difference in gene expression between responders and non-responders was <2 , and the *P* values for the individual gene probe sets corresponding to this gene were not statistically significant.

In addition to these baseline differences, we saw changes in expression of a number of microtubule- and spindle-associated genes when comparing tumor specimens before and after one cycle of chemotherapy. These included spindle assembly checkpoint genes *BUB1* and *BUB3* and *MAD2L1* and a number of tubulin isoforms. These genes may play also an important role in determining response to taxane containing chemotherapy.

We also noted significantly increased expression in a number of genes involved in the DNA repair pathway after one cycle of chemotherapy including *BRCA1*, *PARP2*, and *CHEK1*. DNA damage is the mechanism of action of many chemotherapeutic agents with activity in breast cancer, and alterations in DNA repair pathways are important in the pathogenesis of breast cancers associated with *BRCA1* and *BRCA2* mutations [12]. In addition, a new class of targeted agents, the PARP inhibitors, which inhibit a key enzyme involved in DNA repair, are being evaluated both as monotherapy for *BRCA*-associated cancers and in combination with DNA damaging agents [13, 14]. Given the known overlap between *BRCA1* mutations and triple-negative tumors, these agents are also being evaluated in the setting of triple-negative disease (<http://www.cancer.gov/search/ResultsClinicalTrialsAdvanced.aspx?protocolsearchid=5122624>). About a third of our study patients

had triple-negative tumors, and a large percentage of responders in our study had triple-negative disease. The data from large neoadjuvant chemotherapy studies show that women with triple-negative tumors are more likely to achieve a pathologic complete response than those with hormone sensitive tumors, but those who do not respond have a poorer prognosis [15]. Thus, if DNA repair plays a key role in both the pathogenesis of these tumors, and in their response to treatment, as our data suggest, then the measurement of expression of these genes at the time of diagnosis and early in the course of chemotherapy may be both prognostic and predictive.

Genes in the FGF pathway have recently been shown in genome wide association studies to be related to risk of breast cancer [16, 17], and tumor expression of FGF has been suggested to confer chemotherapy resistance, particularly resistance to paclitaxel [18, 19]. In our analysis, expression of *FGFR1OP* was significantly increased after one cycle of chemotherapy. The product of this gene is a centrosomal protein that is involved in the anchoring of microtubules, and has been implicated as a prognostic indicator in non-small cell lung cancer [20]. Also consistent with this hypothesis, *FGFR1* gene expression decreased in responders in our analysis.

Genes thought to be involved in determining responsiveness to capecitabine, such as thymidylate synthetase, thymidine phosphorylase, and dihydropyrimidine dehydrogenase [21] did not play a key role in our study. Pusztai et al. have recently suggested that small phase II studies are unlikely to identify individual genes that are significantly associated with response to individual therapeutic agents [22]. One obvious challenge is the lack of power in small studies combined with the problem of multiple comparisons, which makes it difficult to distinguish truly differentially expressed genes from noise. Alternatively, significant heterogeneity among tumors in these small studies could lead to the dilution of true differences in gene expression that might be apparent if more homogenous tumors were studied. Our patient population was quite heterogeneous with regard to molecular and histologic characteristics of the tumors. It is becoming increasingly apparent that different subclasses of breast cancer carry very different prognoses, and likely respond differently to particular classes of therapeutic agents. Thus, an analysis of changes in expression of DNA repair genes that is limited to triple-negative tumors may be more relevant to determining outcomes than that involving a mixed group of tumors.

The PAM50 analysis of our baseline specimens revealed a reasonable degree of agreement between ER and PgR expression and subtype classification, such that samples with ER or PgR expression by IHC tended to fall into the luminal categories, while triple-negative tumors expressed

Fig. 3 Heatmap from PAM50 Hierarchic cluster analysis from using (a) all patient specimens (b) baseline specimens only. Sample key: *blue* luminal A, *light blue* luminal B, *pink* HER2-enriched, *red* basal-like, *green* normal-like

the basal phenotype. In addition, though the numbers are extremely small, the PAM50 analysis in paired pre- and post-chemotherapy specimens raises several interesting issues. First, we found that ROR scores generally decreased after one cycle of chemotherapy. This may reflect cell cycle arrest in response to chemotherapy. In addition, we saw that after one cycle of chemotherapy, the assigned phenotype for a majority of our specimens changed, with the most common change being from luminal B to luminal A subtype. These data could reflect a slowing of proliferation of tumor cells, rendering them more similar to the

luminal A subtype. Alternatively, our data could reflect selective killing of tumor cells that are sensitive to chemotherapy, leaving a sub-population of hormone sensitive, less chemotherapy responsive tumor cells. While our results are biologically intuitive, these data must be interpreted with caution due to the small sample size and to methodological challenges in determining subtype membership on new data sets. One must be cautious in interpreting data from analyses in which mean centering of genes is used, particularly with small sample sizes and in cases where the subtype prevalence in the population being

Fig. 3 continued

studied is different from that in the samples originally used to create the algorithm [23]. Re-assaying our samples using RT-PCR may have yielded different results, and may be helpful in future studies. Thus, larger studies with samples available for RT-PCR are needed to determine if a change in molecular subtype with chemotherapy is a true phenomenon with clinical implications.

As previously noted, small phase II studies using high throughput analysis techniques can be limited by two important issues: a high false discovery rate due to the interrogation of a large number of genes, and significant heterogeneity in gene expression resulting in insufficient

power to identify important individual genes. In our analysis, we attempted to overcome these potential problems by looking at biologically relevant gene pathways and categories rather than individual genes, and by using a previously designed categorization and scoring method based on multiple genes that has been shown to correlate with outcomes [8]. Our GO pathway analyses identified 39 GO categories that discriminated between responders and non-responders, and 71 categories with significantly different expression after one cycle of chemotherapy out of a total of 1,666 categories containing genes that met the thresholding criteria. Using a P value of <0.005 for significance, we

would expect that only eight categories would be significantly different for each of these analyses by chance alone.

Skepticism regarding the ability of small phase II studies to identify pharmacogenomic markers of response to chemotherapy [22] is certainly warranted. However, we believe that performing analyses using biologically relevant pathways rather than individual genes is a viable strategy, especially for studies in which there is limited sensitivity for detecting significant differences in expression of individual genes. Thus, the pathway approach represents an important advance in the field of gene expression analysis.

Overall, our study has several distinct strengths. The availability of fresh frozen tissue collected at multiple time points over the course of treatment is a rare and valuable resource. Although we did not have paired samples on all study participants, we do not believe our study sample was biased. There did not appear to be any correlation between having pre- and post-chemotherapy specimens and tumor size, tumor characteristics or response to therapy.

However, several important limitations should be noted including the small sample size and lack of validation set for our findings. As a result, the data presented here must be considered exploratory. As we enter the era of individualized treatment in oncology, studies in which high quality tumor samples are obtained and stored for research and particularly for molecular diagnostics are crucial. In addition, neoadjuvant chemotherapy studies in which tumor samples taken before and after chemotherapy can be interrogated using novel molecular techniques are critical to advancing our understanding of both the biology of breast cancer, and of individual tumor responses to treatment.

In summary, we conclude that a number of biologically plausible pathways, including microtubule and cell spindle structure and function, DNA repair, and growth factor receptors may play a role in determining sensitivity and response to taxane-based chemotherapy. PAM50 analysis in our tumor specimens showed reasonable agreement between IHC classification and molecular subtype, as seen in other studies. In addition, our data suggest that tumor ROR scores decrease in response to chemotherapy, particularly in responsive tumors, although larger studies are necessary to confirm this finding. Gene pathway analysis using GO categories should be further examined as a means of limiting the false discovery rate of gene expression profiling studies. Further comparison of our findings with data from other investigators who examined the association between gene expression and response to therapy will be valuable in determining which particular genes are most important. Confirmation of these findings in larger studies with available tumor specimens will hopefully place us closer to realizing the goal of individualized therapy for breast cancer.

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