

Gene expression profiling and prediction of response to hormonal neoadjuvant treatment with anastrozole in surgically resectable breast cancer

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Abstract Aromatase inhibition (AI) is the most effective endocrine treatment for breast cancer in post-menopausal patients, but a percentage of hormone receptor-positive cancers do not benefit from such therapy: for example, about 20% of patients treated with anastrozole do not respond and it is still impossible to accurately predict sensitivity. Our main goal was to identify a robust expression signature predictive of response to neoadjuvant treatment with anastrozole in patients with ER+ breast cancer. At the same time, we addressed the question of delineating treatment effects and possible mechanisms of intrinsic resistance occurring in non-responder patients. We analyzed the transcriptome of 17 tru-cut biopsies before treatment and 13 matched surgical samples after 3 months

treatment with anastrozole taken from ER+ breast tumors. Molecular profiles were related to clinical response data. Treatment with anastrozole was associated with a decreased expression of genes relating to cell proliferation and an increased expression of genes relating to inflammatory processes. There was also an enrichment of induction of T-cell anergy, positive regulation of androgen signalling, synaptic transmission and vesicle trafficking in non-responders, and of cell cycle inhibition and induction of immune response in responders. We identified an expression signature of 77 probes (54 genes) that predicted response in 100% of our cases. Five of them were able to accurately predict response on an independent dataset ($P = 0.0056$) of 52 ER+ breast cancers treated with letrozole. Ten fixed independent samples from the anastrozole study were also used for RT-qPCR validations. This study suggests that a relative small number of genes analysed in a pre-treatment biopsy may identify patients likely to respond to AI neoadjuvant treatment. This may have practical utility translatable to the clinics. Furthermore, it

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delineates novel mechanisms of intrinsic resistance to AI therapy that could be further investigated in order to explore circumventing treatments.

Keywords Breast cancer · Aromatase inhibitors · Gene expression profiling · Neoadjuvant treatment · Response prediction · Intrinsic resistance

Introduction

Breast cancer represents the most widespread tumor in Western women: incidence in Italy is more than 30,000 new cases per year. Nearly 80% of human mammary carcinomas express estrogen receptor (ER) at clinically significant levels [1]. Estrogen-bound ER induces the expression of peptide growth factors that are responsible for cancer cell proliferation.

In estrogen-dependent breast tumors, control of tumor growth can be achieved by treatment with estrogen antagonists that block the binding of the hormone to ER: tamoxifen has been the predominant treatment for the past two decades [1, 2]. In postmenopausal women, aromatase inhibitors (AIs) have shown to be more effective than tamoxifen as adjuvant therapy [3–6]. Aromatase is the enzyme that synthesizes estrogens by converting C19 androgens to aromatic C18 estrogenic steroids. Experimental results indicate that auto-crine produced estrogen plays a more important role than circulating estradiol in breast tumor promotion: aromatase is expressed in breast tumor tissue (both cancer and surrounding adipose stromal cells) probably at a higher level than in normal breast tissue [7, 8]. In hormone dependent breast cancer, neoadjuvant therapy with third generation AI therapy (anastrozole, letrozole, exemestane) reduces proliferation, causes cell death and produces tumor regression [9, 10]. Although of proven clinical efficacy, such therapies are still associated with non-response in about 20% of cases [11]. Up to now, it is still impossible to accurately predict who will respond to such treatments.

Furthermore, little is known of (i) the effects of anti-aromatase treatment in vivo, (ii) how these effects vary between tumors and (iii) whether the molecular changes fully reflect and are responsible for the clinical response. Preoperative treatment followed by surgical resection provides an excellent opportunity to correlate baseline molecular markers measured in a diagnostic needle biopsy with subsequent response to treatment, and to delineate molecular changes induced by the treatment itself.

A pivotal study on anastrozole treatment in 12 patients identified 298 genes differentially expressed between partial response and progressive disease [12]. Signatures correlated to response to neoadjuvant treatment have been also identified for exemestane, although on a very small patient cohort, and letrozole [13, 14].

Our goals were to (i) examine changes in gene expression with anastrozole therapy and (ii) identify genetic signatures that might distinguish between ER+ post-menopausal breast cancers benefiting from treatment and those not. To elucidate mode of action, we compared the transcriptional profile before and after three months of anastrozole treatment in 13 patients. In order to derive a predictive signature, we analysed global gene expression profiles of 17 ER+ breast cancers before neoadjuvant therapy and associated them to response values. Genes predictive of response were then tested on a publicly available independent dataset [14] and on an independent cohort of fixed tissues.

Methods

Patients

All tumor samples and clinical data were collected with the Fondazione S. Maugeri ethical committee's approval and patients' informed consent. The 17 patients, enrolled into this study between July 2004 to November 2007, were post-menopausal and had breast cancer stage T2 or T3, greater than 2.5 centimeters, any lymph node status and no distant metastasis. All the tumors were HER2/neu- and ER+/PgR+, except 2 that were ER+/PgR- (Table 1). They received neoadjuvant therapy with Anastrozole (ArimidexTM, Astra Zeneca) 1 mg po/die for three months. Clinical response was evaluated by serial tumor clinical examination and mammary ultrasound bidimensional measurements, performed by single operator (LR) before, during and after treatment. Patients with decrease in tumor volume greater than or equal to 30%, according to RECIST criteria [15], were classified as responders.

Microarray hybridization and data processing

Samples were removed by tru-cut biopsy or surgical resection and stored in RNAlater (Ambion Inc., Austin, TX) for processing. Tissues were homogenized and total RNA was isolated using TriReagent (Sigma-Aldrich, St. Louis, MO) and RNeasy Mini Kit (Qiagen, Dusseldorf, Germany). mRNA amplification and labeling, quality control and microarray hybridization were carried out as previously described [16]. Two oligonucleotide glass arrays (Human Whole Genome Oligo Microarray 1x44K or 4x44K, Agilent Technologies, Palo Alto, CA) were utilized for each sample, using the Human Universal Reference Total RNA (Clontech, Palo Alto, CA) as baseline and dye-swap duplication.

Slides were scanned with the Agilent G2505B scanner and the TIFF images loaded into the Feature Extraction

Table 1 Pathophysiological characteristics and percentage of response to therapy of analyzed patients

Patient	Age (years)	Initial size (mm)	Tru-cut			Surgical sample						Stage			Lymph-node		% Response
			ER (%)	PgR (%)	ki67 (%)	Size (mm)	Histotype	Grading	ER (%)	PgR (%)	ki67 (%)	T	N	M	Positive N°	Total N°	
10051*	70	29	80	0	10	25	ILC	2	80	5	5	2	1	0	1	20	14
10054*	52	37	80	80	8	30	ILC	2	60	40	40	1C	1	0	1	14	19
10052*	80	25	80	80	8	20	IDC	2	80	60	5	1C	1	0	1	7	20
10018*	74	30	80	60	8	23	ILC	2	80	20	10	2	1	0	2	15	23
10000*	70	53	70	40	10	40	IDC	3	70	20	8	3	1	0	14	14	24
10028*	73	26	80	70	5	19	IDC	1	80	60	5	2	1	0	2	10	27
10056*	75	39	70	10	15	28	IDC	1	80	10	8	2	0	0	0	22	28
10064*	83	25	80	80	5	18	IDC	1	80	40	5	1C	NV	1	NV	NV	28
10040	72	28	80	80	5	19	ILC	2	80	70	5	1C	NV	0	NV	NV	32
10026*	76	30	80	80	10	20	IDC	2	90	70	10	2	NV	0	NV	NV	33
10010*	66	25	70	90	8	16	ILC	2	80	80	8	1C	0	0	0	8	36
10036	66	25	60	0	5	16	ILC	2	80	0	10	2	0	0	0	8	36
10017*	73	26	90	90	40	16	IDC	2	90	80	10	1C	0	0	0	12	38
10039	85	27	80	80	10	16	ILC	3	80	70	5	1C	NV	0	NV	NV	40
10037	65	25	80	80	5	14	IDC	2	80	70	5	1C	0	0	0	6	44
9999*	78	40	80	70	5	21	IDC	2	80	40	5	2	1	0	8	9	47
10061*	79	27	90	90	10	13	ILC	2	90	80	10	1C	NV	0	NV	NV	52

The * indicates patients with both pre- and post-treatment samples available for hybridization

ILC infiltrating lobular carcinoma, IDC infiltrating ductal carcinoma, NV not valuable

software (Agilent Technologies). Raw data were processed using the statistical computing software “R” and packages from Bioconductor (www.bioconductor.org); “limma” was used for preprocessing and differential expression analysis. Raw intensities were first background corrected (“normexp” method, with an offset value of 50 added to red and green intensities so that log-ratios are shrunk towards zero at lower intensities). In order to set log-ratios average to zero within each array and to have similar log-ratio distributions across all arrays, “loess” and “Aquantile” normalization were performed [17].

Gene ontology analysis was accomplished with GOEAST (<http://omicslab.genetics.ac.cn/GOEAST/>) and pathway analysis with “MetaCore 5.0” (GeneGO, St. Joseph, MI).

Signature extraction and evaluation

Genes differentially expressed by the treatment were obtained as previously described [18]. The difference matrix was defined as the difference between post and pre treatment expression matrices. To derive a predictive signature, we first calculated the Pearson correlation coefficient between tru-cut expression and response data, used as continuous variable. Then the two sample modified *t*-test available in the “limma” package was performed, using response data as a binary variable. The best predictors from the two lists were selected using the rank product method.

For sample classification, leave-one-out cross validation procedure was applied. Briefly, for each list of predictors, the following procedure was carried out: one sample was leaved out and two centroids, one representing responders and the other non-responders, were derived by kmeans clustering and response status was assigned based on majority rule. Then the sample initially leaved out was assigned a class based on Euclidean distance from the two centroids. The procedure was repeated 17 times leaving out each time one sample from centroid calculation and the number of correct prediction reflects the performance of the signature.

To validate the procedure, radical leave-one-out cross validation was performed, where the whole process of signature derivation was repeated leaving each time one sample out of the analysis and the derived signature was used to classify the sample initially leaved out.

The standalone JAVA-based Signature Evaluation Tool (<http://microarray.ym.edu.tw>) was used to predict responsiveness on test samples in radical leave-one-out cross validation and on the independent dataset (GSE5462).

RT-qPCR

Total RNA was extracted from an independent cohort of 10 formalin-fixed, paraffin-embedded breast cancer pre-treatment biopsies using Recover All Nucleic Acid Isolation kit

(Ambion Inc., Austin, TX), according to manufacturer instructions.

Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) was performed as previously described [19] to validate a subset of differently expressed transcripts (CXCL3, GSS, FBXL18 and DUSP1) identified by microarray analysis on fresh samples. Primers sequences are available in Supplementary Table 1. First strand cDNA synthesis was performed using 200 ng of total RNA. The relative abundance of transcripts was established using the mean expression of TBP and RPLP0 for normalization of input cDNA.

Results

A flow chart outlining the statistical methods used for analyzing expression data is shown in Fig. 1.

Change in gene expression with therapy

The molecular profiles of all the 17 patients listed in Table 1 were analyzed before therapy, and for 13 of them (identified by a star after the patient's code) we could also evaluate the change in gene expression with treatment. Three different approaches were used to find probes differentially expressed between pre and post-treatment samples [18]: frequency of change, which measures the consistency by which a probe is up or down-regulated; magnitude of change, which

identifies probes having greatest median fold change across all patients; statistically significant change, which identifies probes most significantly changed with therapy, applying two sample paired Student *t*-test.

Combining the lists from the three methods resulted in 634 down-regulated and 646 up-regulated probes, 78 (47 up and 31 down-regulated) of which were reported differentially expressed by all the three methods (Table 2). Venn diagrams representing overlaps between the three lists are shown in Fig. 2. Hierarchical clustering on 1280 probes using the difference matrix was not able to separate responders from non-responders in a statistically significant manner (Supplementary Figure 1). Differentially expressed probes were compared with a list of 2897 estrogen-regulated genes [20], found modulated in response to estrogens and also present on Agilent 44K array. Hypergeometric test showed that our list is significantly enriched in estrogen-regulated genes (Fisher exact test P value = 2.2×10^{-16}), as revealed by Metacore analysis, too (data not shown).

In order to understand the functional characteristics of genes changed with treatment, gene ontology analysis was performed separately for down and up-regulated probes. The down-regulated list is basically enriched in terms related to cell cycle (Supplementary Tables 8 and 9), as cell cycle phase, M-phase, mitotic cell cycle, cell cycle, mitosis cell division and regulation of mitotic cell cycle. Other overrepresented GO terms were response to DNA damage stimulus, DNA replication and DNA integrity checkpoint. GO analysis of up-regulated genes showed that this list is more heterogeneous and less specific in terms of functions.

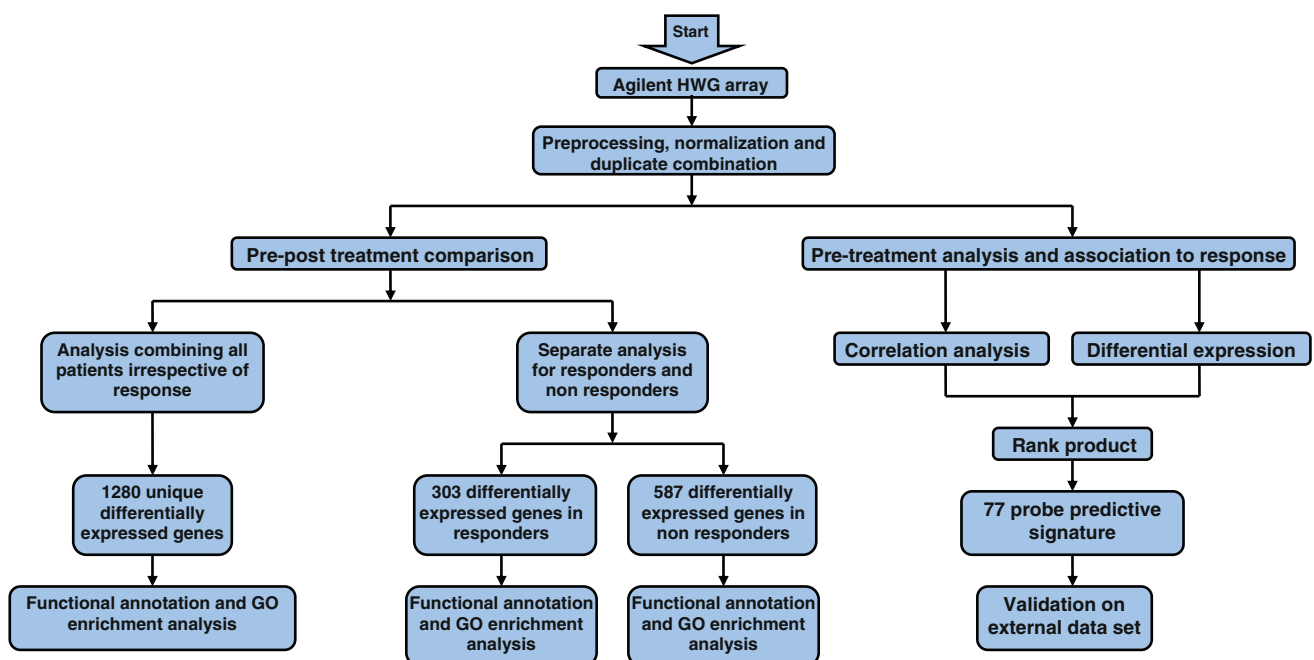


Fig. 1 Flow-chart of data processing and analysis

Table 2 78 probes changed with treatment and found by all three statistical methods

Down-regulated gene description	
Gene symbol	
RAMP	RA-regulated nuclear matrix-associated protein (RAMP)
CDCA5	Cell division cycle associated 5 (CDCA5)
TK1	Thymidine kinase 1, soluble (TK1)
HSPC150	HSPC150 protein similar to ubiquitin-conjugating enzyme (HSPC150)
KIAA0101	KIAA0101
PGR	Progesterone receptor (PGR)
HCAP-G	Chromosome condensation protein G (HCAP-G)
FKSG14	Leucine zipper protein FKSG14 (FKSG14)
THC2217339	Unknown
UHRF1	Ubiquitin-like, containing PHD and RING finger domains, 1 (UHRF1)
BC082981	cDNA clone IMAGE:6575075
GREB1	GREB1 protein (GREB1), transcript variant a
MGC24665	Hypothetical protein MGC24665 (MGC24665)
CDT1	DNA replication factor (CDT1)
CENPF	Centromere protein F, 350/400 ka (mitosin) (CENPF)
SUSD3	Sushi domain containing 3 (SUSD3)
FLJ12650	Hypothetical protein FLJ12650 (FLJ12650)
ASPM	Asp (abnormal spindle)-like, microcephaly associated (Drosophila) (ASPM)
ZWINT	ZW10 interactor (ZWINT), transcript variant 2
RECQL4	RecQ protein-like 4 (RECQL4)
BM039	Uncharacterized bone marrow protein BM039 (BM039)
UBE2C	Ubiquitin-conjugating enzyme E2C (UBE2C), transcript variant 6
C22orf18	Chromosome 22 open-reading frame 18 (C22orf18), transcript variant
RPL23A	UI-H-DH0-aul-h-03-0-UI.s1 NCI_CGAP_DH0 cDNA clone IMAGE:5871026 3', sequence
RAN	UI-H-DH0-aum-e-05-0-UI.s1 NCI_CGAP_DH0 cDNA clone IMAGE:5871340 3', sequence
THC2162693	RS24_HUMAN (P16632) 40S ribosomal protein S24 (S19), partial (77%)
RIMS4	Regulating synaptic membrane exocytosis 4 (RIMS4)
AL137566	cDNA DKFZp586G0321 (from clone DKFZp586G0321)
A_32_P54260	Unknown
FLJ10719	Hypothetical protein FLJ10719 (FLJ10719)
THC2089973	BU589712 AGENCOURT_8908071 NIH_MGC_141 cDNA clone IMAGE:6388605 5', sequence
Up-regulated gene description	
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS)
DUSP1	Dual specificity phosphatase 1 (DUSP1)
TGFB114	Transforming growth factor beta 1 induced transcript 4 (TGFB114), transcript variant 1,
MLL3	cDNA DKFZp686C08112 (from clone DKFZp686C08112)
CTGF	Connective tissue growth factor (CTGF)
PER3	Period homolog 3 (Drosophila) (PER3)
RAB6A	RAB6A, member RAS oncogene family (RAB6A), transcript variant 1
PLDN	Pallidin homolog (mouse) (PLDN)
PLDN	Pallidin homolog (mouse) (PLDN)
SNAP23	Synaptosomal-associated protein, 23 kDa (SNAP23), transcript variant 1
EGR1	Early growth response 1 (EGR1)
A_23_P300563	Unknown
A_23_P31563	Unknown
AL833005	cDNA DKFZp666D074 (from clone DKFZp666D074)

Table 2 continued

	Up-regulated gene description
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1) (PRKAR1A), transcript variant 1
CYR61	Cysteine-rich, angiogenic inducer, 61 (CYR61)
MLL3	Myeloid/lymphoid or mixed-lineage leukemia 3 (MLL3)
CRIM1	Cysteine-rich motor neuron 1 (CRIM1)
A_24_P101072	Unknown
SCP2	Sterol carrier protein 2 (SCP2), transcript variant 1
RHOA	Ras homolog gene family, member A (RHOA)
AF130080	Clone FLC0675] PRO2870
AF130080	Clone FLC0675 PRO2870
RAB7	RAB7, member RAS oncogene family (RAB7)
ATP6V1C1	ATPase, H ⁺ transporting, lysosomal 42 kDa, V1 subunit C, isoform 1 (ATP6V1C1), transcript variant 1
THC2166450	Unknown
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1) (PRKAR1A), transcript variant 1
CYR61	Cysteine-rich, angiogenic inducer, 61 (CYR61)
KIAA0738	KIAA0738 gene product (KIAA0738),
XTP7	Protein 7 transactivated by hepatitis B virus X antigen (HBxAg) (XTP7)
MALAT1	cDNA DKFZp586G1023 (from clone DKFZp586G1023)
AK097068	cDNA FLJ39749 fis, clone SMINT2017599
AA452590	AA452590 zx39f03.s1 Soares_total_fetus_Nb2HF8_9w cDNA clone IMAGE:788861 3',
SON	SON DNA binding protein (SON), transcript variant
PUM2	Pumilio homolog 2 (Drosophila) (PUM2)
RABGEF1	RAB guanine nucleotide exchange factor (GEF) 1 (RABGEF1)
PRG1	Proteoglycan 1, secretory granule, (cDNA clone IMAGE:4688573)
CXorf39	cDNA: FLJ21191 fis, clone COL00104
EIF2C4	For KIAA1567 protein
SMG1	PI-3-kinase-related kinase SMG-1 (SMG1), transcript variant 1
NUDT4	nudix (nucleoside diphosphate linked moiety X)-type motif 4 (NUDT4), transcript variant 2
SMG1	PI-3-kinase-related kinase SMG-1 (SMG1), transcript variant 1
SMG1	PI-3-kinase-related kinase SMG-1 (SMG1), transcript variant 1
LOC286170	cDNA FLJ31058 fis, clone HSYRA2000828
NOTCH2NL	cDNA FLJ11946 fis, clone HEMBB1000709
LOC200030	Hypothetical protein LOC200030 (LOC200030)
AK027225	cDNA: FLJ23572 fis, clone LNG12403

The up-regulated genes feature terms like MHC protein complex, antigen processing and presentation, phosphoinositide phosphorylation, immune system process and insulin like growth factor binding. The top GO processes, pathway maps and network processes found for the up and down-regulated probes with their *P*-values are listed in Supplementary Table 10 and graphically shown in Fig. 3.

Signature predictive of response

Using the response criteria as described in methods, 9 patients were classified as responders and 8 as non-responders (Table 1). No patient showed disease progression during the

treatment. In this limited series of 17 breast cancers it has not been possible to identify any statistically significant association between the response and the base-line expression of ER, PGR, Ki67, or lymph nodes involvement. We only observed that tumors with low PgR and N1 responded less than tumors with high PgR tumors and N0, respectively.

So we applied correlation analysis using pre-treatment expression data and the percentage of response as a continuous variable. The log ratios for each probe across 17 samples and the response data were scaled to have mean zero and standard deviation one. A filter was applied to remove all the probes with more than three missing values or standard deviation less than 0.2 across 17 pre-treatment

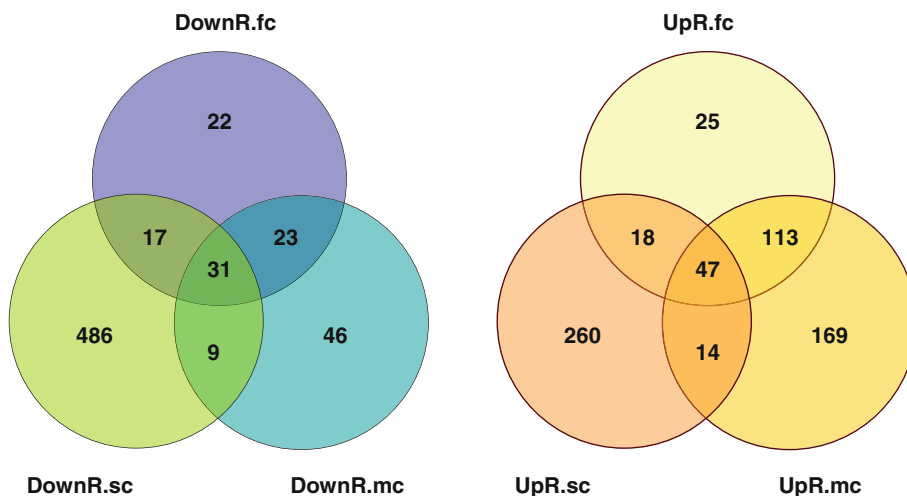


Fig. 2 Overlap among differentially expressed probes. Venn diagram representing overlap among down-regulated probes (a) and up-regulated probes (b) from three methods. Using the frequency of change approach (fc), 93 down-regulated and 203 up-regulated probes in 10 out of 13 patients were found (Supplementary Tables 2 and 3). Using the magnitude of change (mc) method (change in at least 10

cases), we found 109 down-regulated and 343 up-regulated probes (Supplementary Tables 4 and 5). With the statistical significant change (sc) approach, we found 543 down-regulated and 339 up-regulated probes with two sample paired *t*-test adjusted *P*-value less than 0.01 (Supplementary Tables 6 and 7)

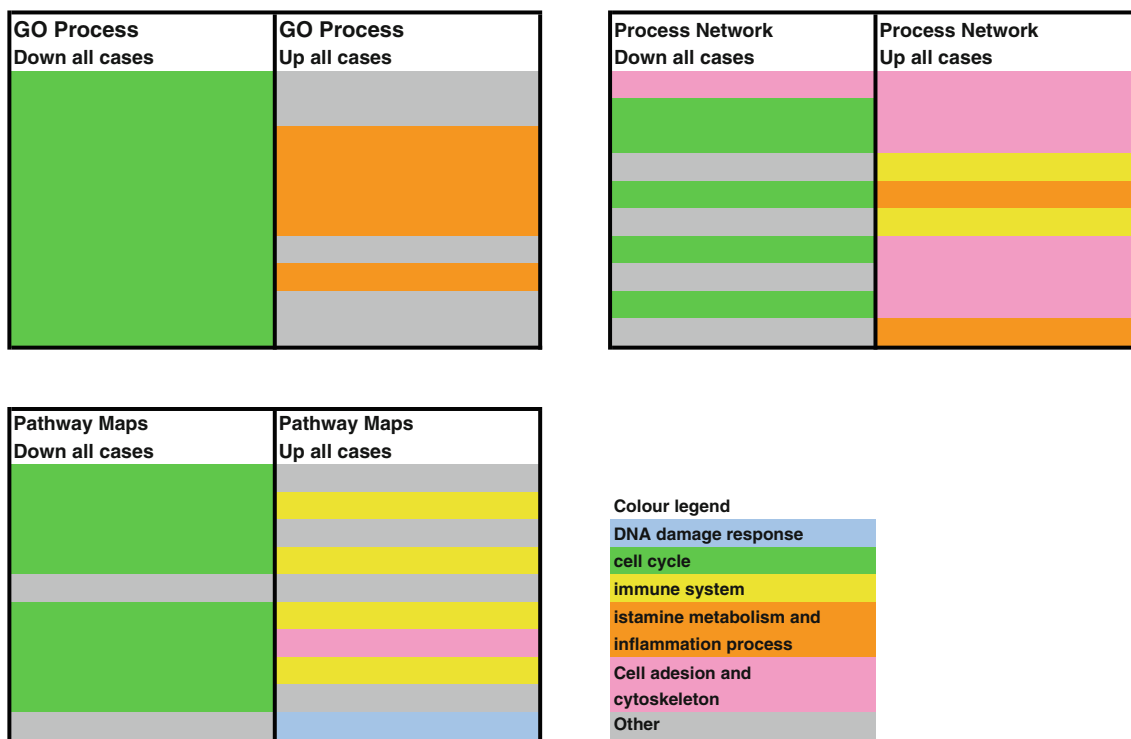
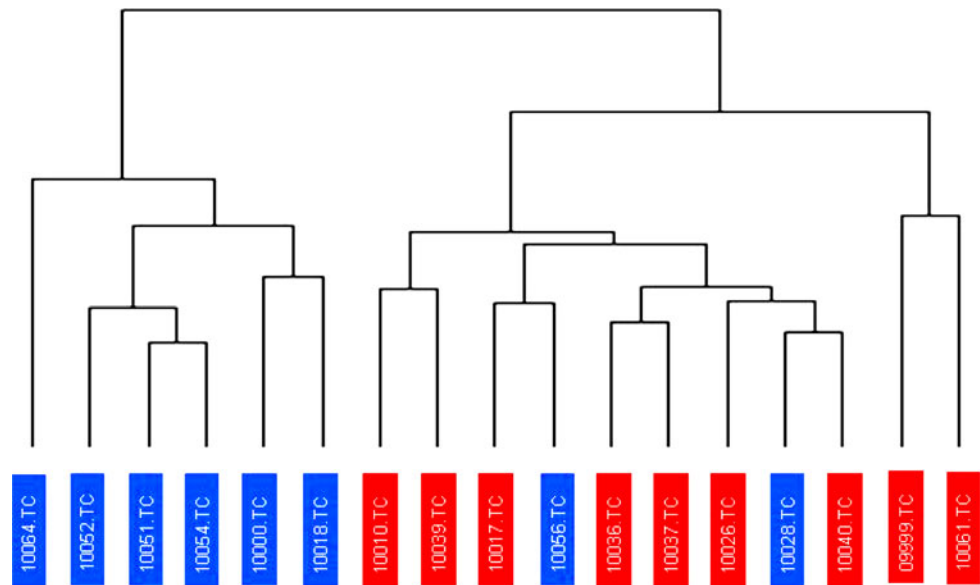


Fig. 3 Go analysis of genes differentially expressed in pre-post treatment samples. Each *rectangular* represents a single category and is ordered according to ascending *P*-value (as in Supplementary Table 10). Similar pathways or processes have the same color code

samples. After the filtering step, 35,181 probes were selected for further analysis. 159 probes had correlation test *P*-value less than 0.01. Out of these, 107 were positively correlated and 52 negatively correlated with response. Probes with their correlation and associated *P*-value are

listed in Supplementary Table 11. Hierarchical clustering with Euclidean distance over 159 probes classifies patients into 2 distinct groups (Fig. 4): the cluster on the left contains only non-responders, while the one on the right contains all the responders and two patients with response

Fig. 4 Hierarchical clustering with Euclidean as distance measure over the 159 probes most correlated with response classifies patients into 2 distinct groups. *Blue rectangle* = non-responders; *red rectangle* = responders



values slightly below the cut-off. Repeated runs of *k*-means clustering find similar grouping when patients are classified in two clusters. Leave-one-out cross validation (with 2-means clustering) correctly predicts responsiveness of 14 patients out of 17 (Fisher *P*-value = 0.015).

Then two Sample *t*-test was performed on pre-treatment samples to identify genes differentially expressed between responders and non-responders. Analysis selected 234 probes with *P*-value < 0.01 (Supplementary Table 12). Hierarchical clustering over these probes separated responders from non-responders with one exception (data not shown). The patients misclassified had response percentage 27 and was actually placed into the responsive cluster group. *k*-Means clustering produced the same results when samples are classified into two groups. Leave-one-out cross validation correctly predicts 16 patients (Fisher *P*-value = 0.0004). The wrongly predicted sample was the same previously misclassified case.

The predictive signatures identified by the two methods have 39 probes in common. In order to have best compromise for informative probes, the two signature lists were combined using the rank product method. Probes were ordered according to the product of their ranks. The top 77 probes were selected as final signature (54 unique genes, Tables 3 and 4), as they performed with 100% accuracy in separating responders from non-responders in leave-one-out cross validation. To validate the procedure, radical leave-one-out cross validation was performed and SET (Signature Evaluation Tool) was used to predict the responsiveness of the test sample. We were able to correctly predict responsiveness for 82% of the samples (Fisher exact *P*-value = 0.015).

The 77-probe signature predictive of response to anastrozole was then tested on a totally independent dataset

(GSE5462) of 52 patients treated with neoadjuvant letrozole therapy [14], using SET. Five genes (in bold in Tables 3 and 4) correctly classify 26 out of 37 responders and 11 out of 15 non-responders (Fisher exact test *P*-value = 0.0056).

qRT-PCR results

Four of those genes (CXCL3, GSS, FBXL18 and DUSP1) were also tested by means of RT-qPCR on a cohort of 10 FFPE breast pre-treatment biopsies from the anastrozole study, that were not used for the gene expression signature derivation. Their expression changes were in accordance with microarray analysis results, although the differences between the two groups (responders and non-responders) were not statistically significant (Supplementary Figure 2).

Changes with treatment in responder and non-responder sub-groups

A separate analysis for responders and non-responders was carried out in order to investigate whether therapy affected the expression in a different manner. The separate analysis showed that a larger number of probes were affected by the treatment in non-responders compared to responders (303 in responders and 587 in non responders). No probe was found oppositely modulated in the two groups. Most of the probes found differentially expressed in one group were not affected by the treatment in the other (only 49 probes were common between two analyses).

GO analysis of the genes differentially expressed in responders, pointed out an enrichment in GO processes, pathway maps and network processes linked to down-regulation of cell cycle, stimulation of the immune

Table 3 Genes which are up-regulated in non-responders compared to responders in pre-treatment samples and also are correlated with response data

	Gene symbol	Up-regulated gene description
1	AGMAT	Agmatine ureohydrolase (agmatinase)
2	ATXN7L2	Ataxin 7-like 2
3	BPA-1	Brain peptide A1
4	C17orf91	Chromosome 17 open reading frame 91
5	C2	Complement component 2
6	CASP4	Caspasi 4
7	COL27A1	Collagen, type XXVII, alpha 1
8	CXCL3	Chemokine (C-X-C motif) ligand 3
9	FOXS1	Forkhead box S1
10	FUNDC2	FUN14 domain containing 2
11	GNRHR	Gonadotropin-releasing hormone receptor
12	H6PD	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
13	HMX2	H6 family homeobox 2
14	INO80D	INO80 complex subunit D
15	KIAA0913	
16	LOC157627	
17	MTA2	Metastasis associated 1 family, member 2
18	NUMBL	Numb homolog (Drosophila)-like
19	OR6Y1	Olfactory receptor, family 6, subfamily Y, member
20	PAX4	Paired box 4
21	RELT	Tumor necrosis factor receptor
22	SFRS15	Splicing factor, arginine/serine-rich 15
23	SPESP1	Sperm equatorial segment protein 1
24	SPN	Sialophorin (CD43)
25	TANC2	Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2
26	ZNF157	Zinc finger protein 157

response, and cell adhesion and cytoskeleton (Fig. 5, right panel, and Supplementary Table 10). On the contrary, non-responders showed an increase in mechanisms of induction of T-Cell energy by MHC antigen presentation without IL-2 production, and were characterized by a positive regulation of processes and pathways related to androgen receptor nuclear signaling, vesicle trafficking and synaptic transmission (Fig. 5, left panel, and Supplementary Table 10).

Discussion

Studies to investigate the effect of estrogen or endocrine agents on gene expression have usually utilized in vitro systems in which cells were cultured in presence of agents for only hours or days before expression analysis [21]. Early responsive genes have been identified whose expression may or may not remain modulated after a longer period of exposure. Considering the complexity of breast tumor, it is important to utilize different approaches to investigate the effect of estrogen on tumor proliferation [22–25]. In this respect, neoadjuvant treatment provides a

unique opportunity to study molecular/genetic changes induced by therapy in breast cancer patients. However, relative few studies have been published so far on pharmacogenomic predictors of response [12–14, 18, 26, 27].

In the present study, we analyzed the molecular changes induced by anastrozole in post-menopausal breast cancer patients and looked for a signature predictive of response. For this purpose, whole genome expression profiles of biopsies collected before treatment were analyzed and associated with clinical–pathological response to treatment.

Gene ontology analysis of differentially expressed genes between pre and post-treatment samples showed that the list of down-regulated genes mainly contains features related to cell cycle progression, growth, DNA damage stimulus and DNA integrity checkpoint. Proliferation promoting genes are also differentially expressed between pre and post-treatment samples. Gene network analysis also showed that many genes transcriptionally activated by ESR1 (estrogen receptor 1) were among the down-regulated list. Similar results for down-regulated genes were also obtained by Miller et al. [18] and Mackay et al. [27].

By contrast, among up-regulated genes we found the overrepresentation of processes linked to immune system,

Table 4 Genes which are down-regulated in non-responders compared to responders in pre-treatment samples and also are correlated with response data

	Gene symbol	Down-regulated gene description
1	ADAMTS4	ADAM metalloproteinase with thrombospondin type 1 motif, 4
2	AGRN	Agrin
3	ARHGAP29	Rho GTPase activating protein 29
4	CDX1	Caudal type homeobox 1
5	COMMD10	COMM domain containing 10
6	CPSF3L	Cleavage and polyadenylation specific factor 3-like
7	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9
8	DUSP1	Dual specificity phosphatase 1
9	FBXL18	F-box and leucine-rich repeat protein 18
10	GABRG2	Gamma-aminobutyric acid (GABA) A receptor, gamma 2
11	GSS	Glutathione synthetase
12	H3F3B	H3 histone, family 3B (H3.3B)
13	KTN1	Kinectin 1 (kinesin receptor)
14	LOC257396	
15	LOC285831	
16	LOC440093	
17	NAPB	N-Ethylmaleimide-sensitive factor attachment protein, beta
18	NAPG	N-Ethylmaleimide-sensitive factor attachment protein, gamma
19	NARS2	Asparaginyl-tRNA synthetase 2, mitochondrial (putative)
20	NSUN4	NOL1/NOP2/Sun domain family, member 4
21	OXCT1	3-Oxoacid CoA transferase 1
22	PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
23	PEX19	Peroxisomal biogenesis factor 19
24	PPP1CC	Protein phosphatase 1, catalytic subunit, gamma isoform
25	SAE1	SUMO1 activating enzyme subunit 1
26	SDCBP	Syndecan binding protein (syntenin)
27	TUBB2C	Tubulin, beta 2C
28	USP13	Ubiquitin specific peptidase 13 (isopeptidase T-3)

histamine metabolism and inflammation, cell adhesion and cytoskeleton, whereas previous reports only pointed out organ development and extracellular matrix turnover. In support of our findings, Wang and coworkers [28] showed that, in a rat model, anastrozole induced increased levels of proinflammatory cytokines and suppressed the differentiation of naive T cells to Treg cells: T-regs inhibit the antitumor functions of tumor-specific T cells by direct cell-to-cell contact.

In general, the comparison between our results on the total cohort and Mackay's gave an overlap of 47 down- and 84 up-regulated common genes, even if Mackay's observations were after 14 days of treatment.

GO analysis of the genes differentially expressed in responders only, revealed enrichment of processes linked to down-regulation of cell cycle and to stimulation of the immune response. On the other hand, non-responders showed an increase in mechanisms of induction of T-cell energy by MHC antigen presentation without IL-2 production: these processes could reduce an immune response versus the tumor cell induced by anastrozole treatment [28].

Furthermore, non-responder tumors showed an up-regulation of network processes related to androgen receptor nuclear signaling: this suggests that tumor resistant cells could divert androgens to another cell proliferation signal to escape from aromatase cell cycle inhibition [29]. Sikora et al. [30] hypothesized that androgens and their metabolites, independently of aromatase activity, may contribute to breast cancer growth. Moreover, non-responders show a down-regulation of negative regulation of synaptic transmission, a mechanism that could be connected to cancer cell proliferation [31] and an up-regulation of Rab-regulated recycling pathways of vesicle: some elements of Rab protein family contribute to breast cancer progression [32, 33].

Our results are the first to demonstrate specific pathways involved into response to AI, with a specific distinction between tumors that respond and those that show intrinsic resistance.

To find a signature predictive of response, probes having maximum correlation with response variable or differentially expressed between responders and non-responders were selected in pre-treatment samples. The differentially

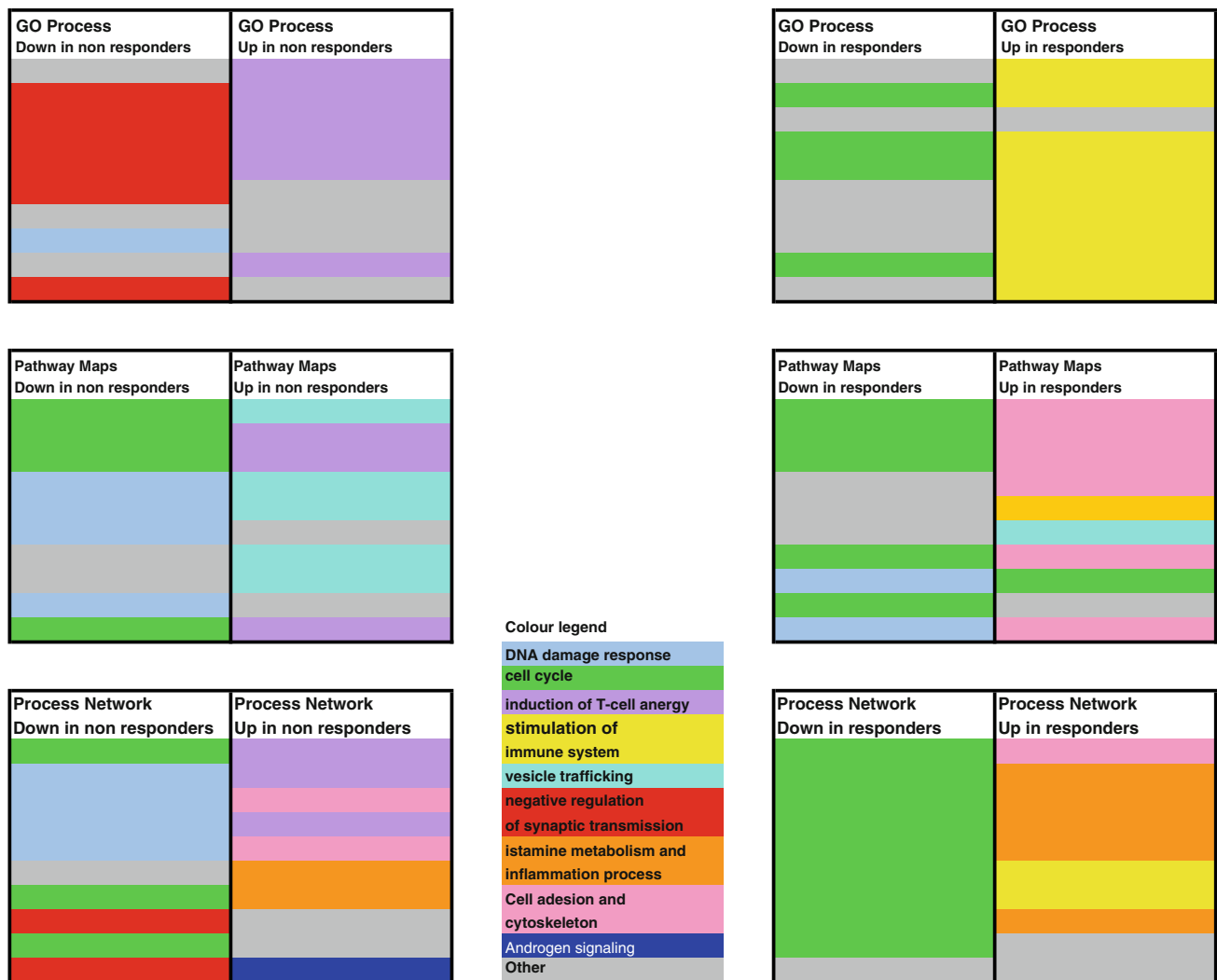


Fig. 5 Go analysis of genes differentially expressed in non-responders, and in responders. Each rectangular represents a single category and is ordered according to ascending *P*-value (as in Supplementary

Table 10). Similar pathways or processes have the same color code. The *right panel* refers to responders while the left one to non-responders

expressed list performed better in separating responders from non-responders. To combine the predictive power of the two lists, probes were arranged according to the product of their ranks and the top 77 were selected as final signature (100% accuracy). In order to test such predictor on an independent cohort, we downloaded the dataset used by Miller and coworkers [14], who derived a signature predictive of response to neoadjuvant treatment with letrozole. Our signature and the 69 pre-treatment variables in Miller's one have no common genes. However, after mapping the 77 unique Agilent probes from our analysis to 66 unique Affymetrix probes on Miller's dataset, we were able to identify six probes predictive of response to letrozole ($p = 0.0056$), by using SET. The six probes correspond to five unique genes, which are CXCL3, GSS, PAX4, KIAA0913, FBXL18. Among these, CXCL3 is up-regulated in non-responders and was previously found

associated with a shorter relapse-free survival of ERalpha-positive breast cancer patients treated with tamoxifen [34].

Only 17 patients satisfying the eligibility criteria discussed above completed the therapy and provided good quality RNA for microarray analysis. Due to the limited number of patients in our cohort, we used them as training-set to derive a signature predictive of response and then used a bigger and totally independent cohort of patients with comparable characteristics as test-set. This was the only larger neoadjuvant series available to best of our knowledge to use as validation set. Although our signature derives from a small patient cohort, it succeeds in predicting response to another AI, very similar to anastrozole, in such a larger independent series.

Four of those genes were also tested on an independent cohort of 10 FFPE biopsies. Dividing patients into two groups, responders and non-responders, we obtained the

same modulation trend as in the previous microarray analysis.

To our knowledge, a limited number of papers have been published on this subject, and none has results comparable to ours. Kristensen et al. [12] used anastrozole too, but analyzed few and heterogeneous (ER+ and ER-) patients, defining only a group of differentially expressed genes between partial response and progressive disease. Harvell et al. [13] used exemestane, alone or in combination with tamoxifen, on 7 ER+ breast cancers and individuated 50 genes associated with response; however, treatment of the 7 patients was not homogeneous: all responders were treated with exemestane only, but among non responders, three were treated with the two drugs in combination and one received exemestane only. Miller and colleagues [14] identified a combination of pre-treatment (biopsy before therapy) and changed-by-treatment (biopsy after 14 days therapy) genes able to predict response on a cohort of 52 samples treated by neoadjuvant letrozole therapy. However, it has to be emphasized that none of the former studies tested their signatures on independent datasets.

If our results will be further validated on a bigger fixed cohort, AI neoadjuvant treatment could be focused on these “likely responder” patients, pointed out by gene expression evaluation of few genes on RNA extracted from fixed biopsy. On the other hand, patients showing a “non-responsive” signature will be operated immediately, reducing risks and costs of an ineffective treatment. Molecular evaluation on tru-cut biopsy only has the advantage of avoiding treatment on patients predicted to be non-responders, without waiting for 14 days and re-biopsing the tumor, as required to translate previous results to a clinical setting.

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