SPANDIDOS PUBLICATIONS

Gene expression profile of residual breast cancer after doxorubicin and cyclophosphamide neoadjuvant chemotherapy

MARIA APARECIDA AZEVEDO KOIKE FOLGUEIRA[1], HELENA BRENTANI[2], DIRCE MARIA CARRARO[2], MATEUS DE CAMARGO BARROS FILHO[1], MARIA LUCIA HIRATA KATAYAMA[1], ANA PAULA SANTANA DE ABREU[3], EDSON MANTOVANI BARBOSA[3], CÉLIA TOSELLO DE OLIVEIRA[3], DIOGO F.C. PATRÃO[2], LOUISE D. MOTA[2], MARIO MOURÃO NETTO[2], JOSÉ ROBERTO FIGARO CALDEIRA[4] and MARIA MITZI BRENTANI[1]

[1]Faculdade de Medicina, Universidade de São Paulo, Disciplina Oncologia (LIM24), São Paulo, SP; [2]Hospital do Câncer A.C. Camargo, São Paulo, SP; [3]Instituto Brasileiro de Controle do Câncer, São Paulo, SP; [4]Hospital Amaral Carvalho, Jaú, SP, Brasil

Received April 16, 2009; Accepted June 12, 2009

DOI: 10.3892/or_00000503

Abstract. In breast cancer patients, primary chemotherapy is associated with the same survival benefits as adjuvant chemotherapy. Residual tumors represent a clinical challenge, as they may be resistant to additional cycles of the same drugs. Our aim was to identify differential transcripts expressed in residual tumors, after neoadjuvant chemotherapy, that might be related with tumor resistance. Hence, 16 patients with paired tumor samples, collected before and after treatment (4 cycles doxorubicin/cyclophosphamide, AC) had their gene expression evaluated on cDNA microarray slides containing 4,608 genes. Three hundred and eighty-nine genes were differentially expressed (paired Student's t-test, pFDR<0.01) between pre- and post-chemotherapy samples and among the regulated functions were the JNK cascade and cell death. Unsupervised hierarchical clustering identified one branch comprising exclusively, eight pre-chemotherapy samples and another branch, including the former correspondent eight post-chemotherapy samples and other 16 paired pre/post-chemotherapy samples. No differences in clinical and tumor parameters could explain this clustering. Another group of 11 patients with paired samples had expression of selected genes determined by real-time RT-PCR and CTGF and DUSP1 were confirmed more expressed in post-as compared to pre-chemotherapy samples. After neoadjuvant chemotherapy some residual samples may retain their molecular signature while others present significant changes in their gene expression, probably induced by the treatment. CTGF and DUSP1 overexpression in residual samples may be a reflection of resistance to further administration of AC regimen.

Correspondence to: Dr Maria Aparecida Azevedo Koike Folgueira, Faculdade de Medicina da Universidade de São Paulo, Departamento de Radiologia, Disciplina de Oncologia, Avenida Dr Arnaldo, 455, 4˚ andar, sala 4112, CEP: 01246-903, São Paulo, SP, Brasil
E-mail: makoike@lim24.fm.usp.br

Key words: breast neoplasms, gene expression profiling, doxorubicin, drug resistance, neoadjuvant therapy, microarray analysis, reverse transcriptase polymerase chain reaction

Introduction

Most breast cancer patients are offered chemotherapy, as an improvement in 10-year survival between 2-11% was already demonstrated. Primary chemotherapy is associated with the same survival benefits as adjuvant chemotherapy, with the advantage of an increased likelihood of breast conservation (1,2). However, even though a high clinical objective response rate is reported by most authors, only a small pathological complete response (pCR) rate is observed, after primary chemotherapy based in doxorubicin (1,3). Cancer response to chemotherapy may vary from partial clinical response to pathological complete response. Incomplete response may involve either the selection of a resistant clone after disappearance of the sensitive tumor cells or the development of new cellular characteristics during tumor progression, through the expression of other genes, upon chemotherapy stress.

Residual tumors represent a clinical challenge, as they may be resistant to additional cycles of the same drugs. In accordance with this hypothesis, it was demonstrated that upon partial clinical response after four cycles of multi-agent chemotherapy, including doxorubicin, patients randomized to receive a taxane doubled their chance of attaining a pCR, as compared to those randomized to four more cycles of the same treatment (4). Patients submitted to eight cycles of doxorubicin had a similar pCR to those reported by other authors (1-4), after four cycles of neoadjuvant anthracycline (AC).

Our aim was to identify the differential transcripts between residual (post-chemotherapy) and pre-chemotherapy tumor samples from patients considered partially responsive to

primary chemotherapy. This panel of expressed genes may represent potential target genes involved with resistance to AC chemotherapy.

Patients and methods

Patients with histopathologically confirmed invasive breast cancer on samples obtained by core or incisional biopsy were enrolled in a study of gene expression profile associated with response to primary chemotherapy based in doxorubicin, in a routine treatment basis protocol. This study was approved by the Brazilian National Ethics Committee (CONEP, Comitê Nacional de Ética em Pesquisa) and a written informed consent signed by all participants. Patients were prospectively accrued in three reference centers for cancer treatment in São Paulo State, Brazil: Hospital do Câncer A. C. Camargo and Instituto Brasileiro de Controle do Câncer, São Paulo, and Hospital Amaral Carvalho, Jaú, from April 2002 to December 2007.

Sixteen patients had tumor samples collected before treatment, during tumor biopsy, as well as after the four courses of chemotherapy, during the breast surgical procedure. Median age of these patients was 51 years (34-65) and five of them were clinically staged as II, ten as III, and one as IV (supraclavicular node only) (AJCC, 1997). Breast and palpable lymph nodes were considered target lesions and their mean dimension before chemotherapy was 54.1 mm. Fourteen and two patients were diagnosed with invasive ductal carcinomas and invasive lobular carcinomas, respectively. Most tumors expressed estrogen receptor (13/16).

All 16 patients received primary chemotherapy consisting in four cycles of cyclophosphamide 600 mg/m^2 and doxorubicin 60 mg/m^2, every 21 days (AC). Median duration of chemotherapy was 69 days and mean administered dose of doxorubicin was 96.9%. Median interval between the last cycle of chemotherapy and clinical evaluation was 27 days and surgery followed within a median time of 36 days from the last cycle of chemotherapy. Response to chemotherapy was based on RECIST guidelines (5). All the 16 patients were classified as partially responsive and mean percentage of target lesion reduction was 60%. Thirteen patients had involved lymph nodes after treatment, as evaluated by histopathological examination.

Another 11 patients had their tumors subsequently analyzed by real-time RT-PCR to validate gene expression data. Median age of these patients was 53 years (34-70), 64% of them presented clinical stage (CS) III and 36% CS II disease. Mean breast lesion dimension was 48 mm. All tumors were diagnosed as invasive ductal carcinoma, 4/11 expressed estrogen receptor (immunohistochemistry). All patients received four cycles of AC, median duration of chemotherapy was 71 days, mean administered dose of doxorubicin was 96% and surgery followed, after a median interval of 35 days from the last cycle of chemotherapy. All patients were considered responsive to chemotherapy and mean percentage breast lesion reduction was 60%. Five of them presented involved nodes after chemotherapy, as determined by the histopathological examination.

cDNA microarray hybridization and analysis. A cDNA microarray platform containing 4608 open reading frame expressed sequence tags (ORESTES) (6) was assembled at the Ludwig Institute for Cancer Research, São Paulo, Brazil. ORESTES privileges the central part of mRNA molecules and selection of those to be spotted on the slides followed all these criteria: i) cDNA clones representing full length genes; ii) >300 bp and a high quality sequence (CG content); iii) 100 bp region with gene identity >85% as verified on the site http://ncbi.nlm.nih.gov/Blast; iv) cDNA clone 3' sequence. cDNA clones were derived from human breast, colon, stomach, and head and neck tumors. These sequences could be classified among 505 function categories (biological process). Another 192 reference sequences were included as positive and negative controls of hybridization. Platform characteristics complying with MIAME format may be verified in the Gene Expression Omnibus (GEO) data repository, under accession number GPL 1930 (www.ncbi.nlm.nih.gov/projects/geo). This platform was used in previous works of the group (7-10), with consistent results.

Samples obtained from tumor biopsies (pre-chemotherapy) or during breast surgery (post-chemotherapy) were hand dissected and samples with at least 80% tumor cells were further processed. Total RNA was isolated using TRIzol reagent (Invitrogen Co., Carlbad, CA, USA) and RNA quality and integrity was verified by the absorbance $A_{260/280}$, which was >1.8, and through observation of 28S/18S rRNA on agarose gel (1%) electrophoresis in denaturant conditions (ratio >1.0). A two-round RNA amplification procedure was carried out, followed by a reverse transcriptase reaction in the presence of Cy3- or Cy5-labeled dCTP (GE Healthcare Life Sciences, Little Chalfont, St. Giles, UK). HB4A normal epithelial mammary cell line, donated by Drs Mike O'Hare and Alan Mackay (LICR-UCL, London, UK), was used as reference sample for hybridizations. Equal amounts of breast tumor specimens and HB4A cells cDNA labeled probes were concurrently hybridized against cDNA microarray slides. Dye swap was performed for each sample analyzed, to control for dye bias. Reproducibility of hybridization results was revealed by a high correlation index (>0.85) between quantified signals of dye swap samples.

Hybridized arrays were scanned on a confocal laser scanner Arrayexpress (Packard BioScience Co., Boston, MA, USA), using identical photomultiplier voltage for all slides and data recovered by Quantarray software (Packard BioScience), using histogram methods. Saturated spots (signal intensity >63,000) as well as low-intensity spots (within the 95% percentile of intensity distribution of known empty spots) were removed from the analysis. Average signal intensity between technical replicates was determined for each spotted sequence. Quantified signals were then submitted to log transformation and to Lowess normalization.

Paired Student's t-test was used to evaluate the data (matched pre- and post-chemotherapy samples) to avoid bias due to interindividual differences. Discriminatory genes were those satisfying an adjusted p-value <0.01 and false discovery rate (FDR) <0.01 (11). Unsupervised hierarchical clustering analysis based on Euclidian distance and complete linkage was performed using the genes differentially expressed. The reliability of the clustering was assessed by Bootstrap technique implemented in TMEV software (12). All replicates, except one (Q17), clustered together in the dendrogram,

ene specific primers.

Gene name	Genebank accession number	Primer sequence (5'-3')	Amplicon size (bp)
ACTB	NM_001101.2	S - AGAAAATCTGGCACCAACC	188
		AS - AGAGGCGTACAGGGATAGCA	
RPLP0	NM_053275.3	S - GGCGACCTGGAAGTCCAACT	149
		AS - CCATCAGCACCACAGCCTTC	
AXUD1	NM_033027.2	S - CTGGGTAGGGCTGTAGGAAG	247
		AS - CGCTTCTCTTTGGCTGAGTT	
C20orf45	NM_016045.1	S - AACAGATGCAGGGCCTCTAA	186
		AS - TCAGGTGGAGTTCCAATGTATG	
CKLFSF4	NM_181521.2	S - CACAGTTCAAGGACCCATCA	160
		AS - GAGTCCAGGGATGTGGACAG	
CTGF	NM_001901.1	S - CCGTACTCCCAAAATCTCCA	211
		AS - GTAATGGCAGGCACAGGTCT	
DUSP1	NM_004417.2	S - TCCCGACGACACATATACA	167
		AS - TGCTGAAAACAAACCTGCTT	
ITPKC	NM_025194.2	S - AGGCCCCAGAGGTAGCCGTC	170
		AS -GACCCACGAGGTGGTAGGCA	
PLK3	NM_004073.2	S - CTACATGGAGCAGCACCTCA	160
		AS - GTGGTCCCCGTAGAAGTTCA	
SRPRB	NM_021203.2	S - GACAACAGGCAGACTCCACA	161
		AS - TGAACTTCAGAGGCAACTGG	

S, sense; AS, anti-sense.

indicating suitable correction of the individual dye incorporation efficiency by normalization procedure and high experimental reproducibility.

Real-time RT-PCR. To confirm results of gene expression evaluated by cDNA microarray, real-time RT-PCR analysis for some selected genes was performed. Total RNA (2 μg) was reverse transcribed using oligo (dT) primer (GE Healthcare Life Sciences) and Superscript III (Invitrogen Co.). Real-time RT-PCR was carried out using SYBR-Green I (Sigma, St. Louis, MO, USA) in a Rotor-gene system (Corbett Research, Mortlake, Australia). Primer sets were designed based on the coding region closer to the 3' end of the gene using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table I). Sequences present in different exons preferentially separated by long introns were selected, according to sequences deposited at http://www.ncbi.nlm.nih.gov/nucleotide. To avoid non-specific product formation, BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out. To minimize self and cross dimer hairpin formation, homodimer melting temperatures were verified using the program OligoTech version 1.00, Copyright (1995) (Oligos Etc. Inc. & Oligo Therapeutics Inc.).

All samples were tested in duplicates. Cycling conditions were 95˚C for 5 min, followed by 40 cycles at 95˚C for 20 sec, 60˚C for 15 sec and 72˚C for 20 sec. PCR reactions were analyzed by the software Rotor-Gene 6 System (Corbett Research). Average values were used for quantification. HB4a cell line was used as reference.

Expression of five housekeeping genes (ACTB, GUSB, PPIA, RPLP0 and TFRC) was first tested in 15 breast cancer samples and ACTB and RPLP0, which were the most stable among samples, were further used as reference genes to calculate a normalization factor for each sample analyzed, using the geNorm software tool (available at http://medgen.ugent.be/~jvdesomp/genorm/) (13) (data not shown). Relative expression ratio was calculated from the real-time PCR efficiencies and the crossing point deviation of an unknown sample versus a control divided by the normalization factor (14).

Shapiro-Wilk test was employed to verify the distribution pattern of the variables and paired Student's t-test or Wilcoxon signed ranks test, as appropriate, were subsequently utilized to determine the significance level of the difference between groups. A two-sided $p \leq 0.05$ was considered significant. SPSS software version 11.0 (SPSS, Chicago, IL, USA) was utilized.

Results

We have compared the gene expression pattern of 16 paired samples before and after chemotherapy from tumors considered responsive to primary chemotherapy. Three hundred and eighty-nine genes were differentially expressed between post- and pre-chemotherapy samples, 217 over-expressed and 172 down-expressed in post-chemotherapy samples. Expression of up- and down-regulated genes in post-chemotherapy samples varied from 1.09- to 5.50- and 1.13- to 2.64-fold, respectively. Among the differentially expressed

Table II. Genes more expressed in post-chemotherapy samples (pFDR<0.01 and Fc ≥2).

Genes more expressed in post-CT samples	Fc (Post/Pre-CT)
DUSP1 (dual specificity phosphatase 1)	5.5
CTGF (connective tissue growth factor)	3.7
SEPT1 (septin 1)	3.4
FOSB (FBJ murine osteosarcoma viral oncogene homolog B)	3.0
PLK3 [polo-like kinase 3 (*Drosophila*)]	2.9
SMOC2 (SPARC related modular calcium binding 2)	2.8
MGC12972 (hypothetical protein MGC12972)	2.6
BTG2 (BTG family, member 2)	2.5
NAPA (N-ethylmaleimide-sensitive factor attachment protein, α)	2.5
TOM1L2 [target of myb1-like 2 (chicken)]	2.4
FXYD6 (FXYD domain containing ion transport regulator 6)	2.3
NXN (Nucleoredoxin)	2.3
SGK (serum/glucocorticoid regulated kinase)	2.3
STARD13 (START domain containing 13)	2.3
ABI3 (ABI gene family, member 3)	2.2
AXUD1 (AXIN1 up-regulated 1)	2.2
MGC3047 (hypothetical protein MGC3047)	2.2
NPR2 [natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)]	2.2
WWP2 (Nedd-4-like ubiquitin-protein ligase)	2.2
C3 (complement component 3)	2.2
D2S448 (Melanoma associated gene)	2.1
MAP4 (microtubule-associated protein 4)	2.1
NPD007 (NPD007 protein)	2.1
PDGFRL (platelet-derived growth factor receptor-like)	2.1
PPGB [protective protein for ß-galactosidase (galactosialidosis)]	2.1
ADAMTS1 [a disintegrin-like and metalloprotease (reprolysin type) with (thrombospondin type 1 motif, 1)]	2.1
C6orf37 (chromosome 6 open reading frame 37)	2.0
CYFIP2 (cytoplasmic FMR1 interacting protein 2)	2.0
GNAI2 [guanine nucleotide binding protein (G protein), α inhibiting activity (polypeptide 2)]	2.0
PDZRN3 (PDZ domain containing RING finger 3)	2.0
RGS2 (regulator of G-protein signalling 2, 24 kDa)	2.0
SALL2 [sal-like 2 (*Drosophila*)]	2.0
SASH1 (SAM and SH3 domain containing 1)	2.0
SLIT3 [slit homolog 3 (*Drosophila*)]	2.0

Fc (Post/Pre-CT), fold change of mean relative expression between post- and pre-chemotherapy samples.

genes in post-chemotherapy samples, 60 genes presented at least a 2-fold variation between pre- and post-chemotherapy samples, including 34 more (Table II) and 26 less (Table III) expressed genes after chemotherapy.

To identify whether specific functions were regulated, our data were searched considering the biological processes in which differentially expressed genes were involved against all sequences spotted on the slides (http://vortex.cs.wayne.edu:8080/index.jsp) (15) and a corrected p-value <0.05 was considered significant, if at least three genes, involved in that specific function, were represented on the slides (reference ≥3), in order to avoid artifactual results. Six functions were considered modulated, including JNK cascade and cell death (Table IV).

Unsupervised hierarchical clustering identified two branches, one of them comprising eight pre-chemotherapy samples exclusively (Fig. 1). The second one included 8 post-chemotherapy samples (whose pair was present in the first cluster) and another 16 samples (eight paired pre- and post-chemotherapy). No differences in clinical stage, menopausal status, tumor histology, histological grade, estrogen and progesterone receptor or ErbB2 immuno-expression could be associated with this clustering. In addition, a similar tumor reduction (60%) after chemotherapy was detected in samples included in both clusters.

Eight transcripts were selected for further evaluation by real-time RT-PCR based on the following criteria of cDNA microarray analysis: fold change of mean gene expression

Genes less expressed in post-chemotherapy samples (pFDR <0.01 and Fc ≥2).

Genes less expressed in post-CT samples	Fc (Pre/Post-CT)
C20orf45 (chromosome 20 open reading frame 45)	2.6
MRPS17 (mitochondrial ribosomal protein S17)	2.5
CSE1L [CSE1 chromosome segregation 1-like (yeast)]	2.5
BET1 [BET1 homolog (*S. cerevisiae*)]	2.5
ITPKC (inositol 1,4,5-trisphosphate 3-kinase C)	2.5
KLK2 (kallikrein 2, prostatic)	2.4
ZNF19 [zinc finger protein 19 (KOX 12)]	2.4
GLCE (glucuronyl C5-epimerase)	2.4
GIT1 (G protein-coupled receptor kinase-interactor 1)	2.3
SRPRB (signal recognition particle receptor, B subunit)	2.3
TA-PP2C (T-cell activation protein phosphatase 2C)	2.3
CKLFSF4 (chemokine-like factor super family 4)	2.3
RNP24 (coated vesicle membrane protein)	2.3
LOC84661 (dpy-30-like protein)	2.3
C14orf120 (chromosome 14 open reading frame 120)	2.2
PBXIP1 (pre-B-cell leukemia transcription factor interacting protein 1)	2.2
VLDLR (very low density lipoprotein receptor)	2.2
PTBP2 (polypyrimidine tract binding protein 2)	2.1
SPAG9 (sperm associated antigen 9)	2.1
KCTD15 (potassium channel tetramerisation domain containing 15)	2.1
METTL3 (methyltransferase like 3)	2.1
GALC [galactosylceramidase (Krabbe disease)]	2.1
CNNM3 (cyclin M3)	2.1
LMAN2 (lectin, mannose-binding 2)	2.0
ARHE (ras homolog gene family, member E)	2.0
ZNF189 (zinc finger protein 189)	2.0

Fc (Pre/Post-CT), fold change of mean relative expression between pre- and post-chemotherapy samples.

Table IV. Functional categories modulated in post-chemotherapy (CT) as compared to pre-CT samples.

Biological process	Genes over-expressed in post-CT	Genes under-expressed in post-CT	R
Cell recognition	ChGn	CSPG2	3
JNK cascade	PAK1, MAP3K12	DUSP10	11
Protein-nucleous import, docking	XPO6	CSE1L, IPO7	8
Telomerase-dependent telomere maintenance	TNKS1BP1	MRE11A	3
Negative regulation of microtubule depolymerization	KATNB1, MAP4	MAPT	3
Cell death	FAF1, FOSL2		4

Biological processes in which differentially expressed genes were involved against all sequences spotted on the slides were searched for (http://vortex.cs.wayne.edu:8080/index.jsp.) (corrected p-value <0.05). Functions which had at least three genes classified under that specific GO annotation (R, Reference) spotted on the slides, are shown.

between pre- and post-chemotherapy samples >2 and bit error rate (BER) <0.5, which were AXUD1, C20orf45, CKLFSF4, CTGF, DUSP1, ITPKC, PLK3 and SRPRB. As a technical validation, we have first tested the correlation of gene expression evaluated by cDNA microarray and real-time RT-PCR, using the same samples already used. However,

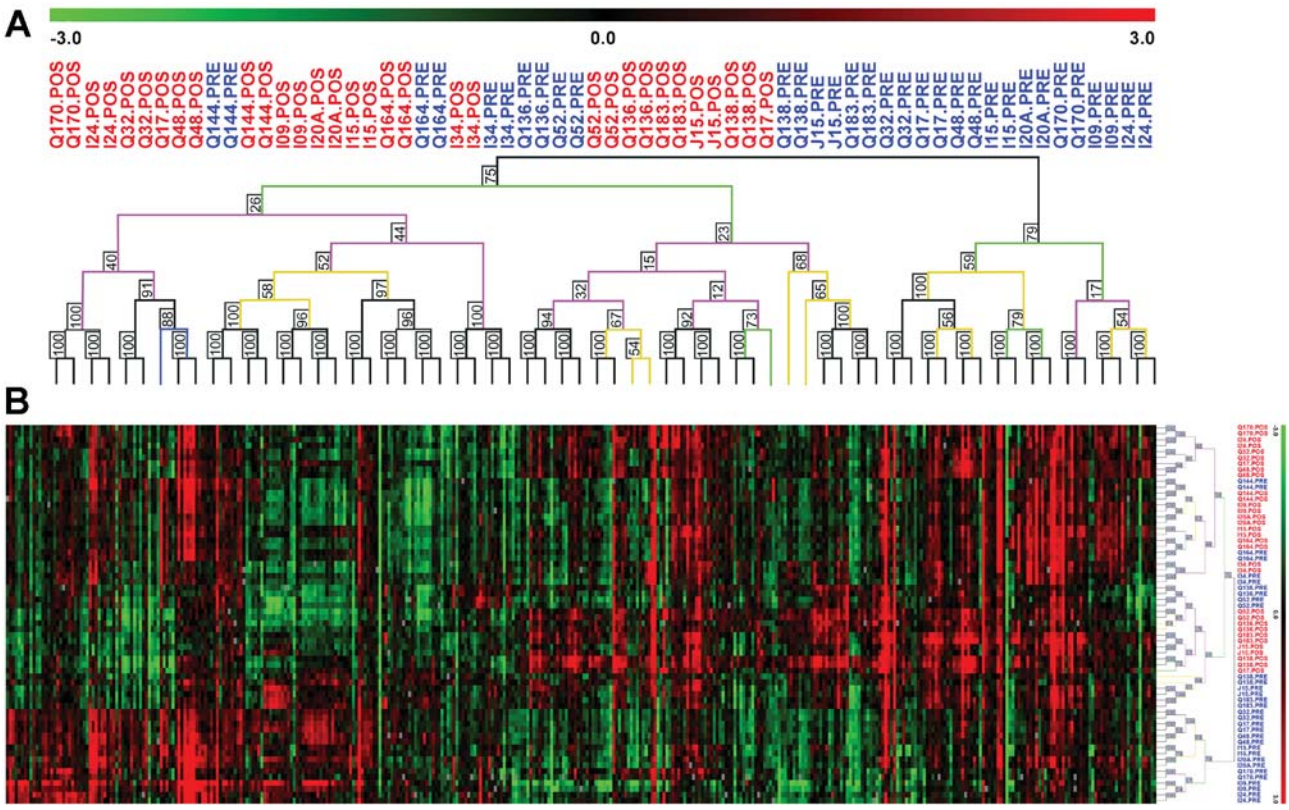

Figure 1. Unsupervised hierarchical clustering of pre- and post-chemotherapy samples. (A) Tumor identification (I, J, Q) appears at the top of the figure, Pre-CT sample (blue) and Pos-CT sample (red). Colored lines of the dendrogram and numbers stand for the support for each clustering, black and gray, more reliable; yellow and red, less reliable. (B) Sixteen paired samples had gene expression evaluated by cDNA microarray. Experiments were made in duplicate (dye swap) and each line represents a tumor sample. Gene expression is represented on columns and the colored bar on the right hand side indicates gene expression variation in target samples as compared to reference cells (HB4A), i.e., red, more expressed and green, less expressed in target samples. Two clusters are clearly seen.

Table V. Correlation of gene expression values evaluated by cDNA microarray and real-time RT-PCR.

	r	P-value
AXUD1	0.719	**0.013**[a]
C20orf45	-0.063	0.846[a]
CKLFSF4	0.552	0.098[a]
CTGF	0.599	**0.040**[b]
DUSP1	0.735	**0.006**[b]
ITPKC	-0.077	0.812[a]
PLK3	0.273	0.391[a]
SRPRB	0.692	**0.013**[a]

As a technical validation, 12 tumor samples (six paired pre- and post-chemotherapy) had their gene expression evaluated by the two techniques. Shapiro Wilk test was used to evaluate normality of the values and Pearson[a] or Spearman rank correlation[b] coefficient (r) and significance level (P), as appropriate, are shown. Positive correlation was observed for six of the genes analyzed and significant correlation for four of them (bold type).

material from only six paired samples was still available for RT-PCR testing. A positive correlation coefficient was observed for six of the eight genes tested and a significant positive correlation for four of them; CTGF, SRPRB, DUSP1 and AXUD1 (Table V).

Expression of these target genes was then determined in samples of another 11 patients submitted to neoadjuvant chemotherapy. In this new set of tumors, DUSP1 and CTGF were confirmed more expressed in post- as compared to pre-chemotherapy samples (Fig. 2). In addition, there was a trend towards a lower expression of C20orf45 and higher expression of AXUD1 in post-chemotherapy tumors, similarly to results obtained previously by cDNA microarray in the first group patients.

Discussion

We have previously observed that gene expression profile of breast cancer samples segregate them according to response to neoadjuvant chemotherapy (responsive vs. non-responsive) (7). In this study we set out to compare gene expression profile from matched samples from patients with partial response to four cycles of AC. Co-aggregation of 50% of the paired (pre- and post-chemotherapy) samples upon cluster analysis suggests that an individual dominant profile was maintained in those tumors. Otherwise, half of the patients (eight) had tumors that clustered in opposite branches, indicating that gene expression pattern could distinguish these pre- and post-chemotherapy samples. Such behavior is not easy to explain,

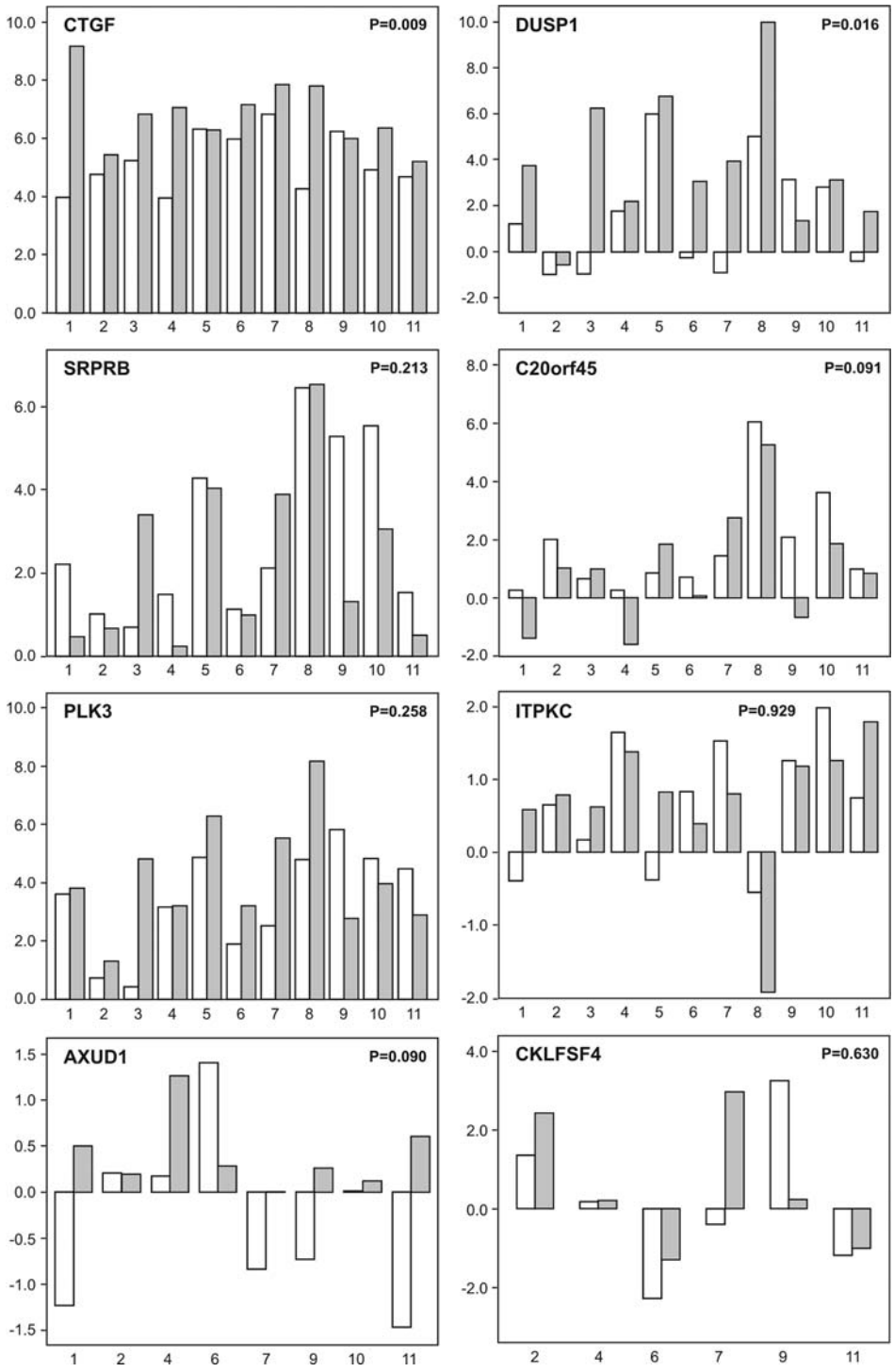

Figure 2. Relative gene expression as evaluated by real-time RT-PCR assays. Eleven paired samples before (Pre-CT) and after (Post-CT) neoadjuvant chemotherapy were evaluated, except for AXUD1 (n=8) and CKLFSF4 (n=6) expression, as a matter of insufficient material for all reactions. Gene expression was verified by real-time RT-PCR and calculated as relative values, as described in Materials and methods. Results were transformed to their $\log_2$ values and each one is shown (white bar, pre-CT; gray bar, post-CT). Shapiro-Wilk test was employed to verify the normality of the results and paired Student's t-test or Wilcoxon signed ranks test, as appropriate, subsequently utilized. P-values appear inside the box. x-axis, samples; y-axis, gene relative expression ($\log_2$ values).

as some residual tumors showed a gene expression profile similar to that observed before treatment and some of them, a differential one, which may indicate clone selection or drug induction.

Previous data demonstrate that the majority of pre- and post-chemotherapy samples co-aggregate upon hierarchical cluster analysis, revealing a high degree of similarity concerning the gene expression profile of samples obtained from the same patient, regardless of treatment. In some reports (16-18) post-chemotherapy samples were collected after a short period of time, varying from 24 h to 21 days after the first cycle, and changes in gene expression might

reflect an early gene modulation by the chemotherapy agents, as it is too early to define a resistance phenotype, following only one course of treatment. It was also suggested that samples that cluster together, independent of chemotherapy, are those from patients with stable disease (19) and samples that cluster in different branches, are those from responsive patients (20). However, even in these two reports, this statement was not universally applicable.

Another proposal to explain this differential pattern of tumor clustering would be that variations in proportional stromal cell content, before and after chemotherapy have occurred. This factor however, probably had little influence on our results, as both pre- and post-chemotherapy samples were mainly represented by tumor cells (80%), after hand dissection and microscopic analysis. In addition, it was shown that despite differences in non-tumor content between paired tumor samples (before and after chemotherapy) and a significant effect exerted by the stromal component on gene expression profiles, tumor samples co-aggregate on cluster analysis, indicating a dominant 'tumor profile' (18).

It was shown that basal and luminal epithelial cell lines have unique transcriptional responses to doxorubicin (21) and that highly tumorigenic breast cancer cells induce a transcriptional response to doxorubicin that is distinct from less malignant cells (22). These hypotheses, however, could not explain the clustering pattern of the samples analyzed, as only one sample presented a triple negative basal phenotype (I15, Fig. 2) and no differences in clinical stage, menopausal status, tumor histology, histological grade, estrogen and progesterone receptors or ErbB2 immune-expression were verified among the samples included in the two branches.

Genes involved in DNA-damage and or stress responses were previously associated with resistance to DNA-interactive drugs (23) and some genes involved in this function were over-expressed in post chemotherapy samples as DUSP1, SGK, BTG2, CTGF, and C3. BTG2 (B-cell translocation gene 2) is a p53 mediated DNA damage response gene, which is up-regulated shortly after the beginning of anthracycline/cyclophosphamide neoadjuvant chemotherapy for primary breast cancer (16). Its over-expression was also demonstrated in MCF-7 subclones intrinsically resistant to DNA-damage (24). Additionally, BTG2 and AXUD1 (axin1 up-regulated), another gene more expressed in our post-chemotherapy samples, were up-regulated in cardiac cells during heart dysfunction induced by doxorubicin chronic use, through free radical generation (25).

In accordance with our data, CTGF (connective tissue growth factor) was previously reported as more expressed in post-chemotherapy samples from both luminal, as well as basal breast cancer samples (21). Moreover, CTGF as well as DUSP1, were found over-expressed in cell subclones with strong bone metastatic potential, suggesting that chemotherapy may be selecting aggressive cell subpopulations (26).

Dual-specificity phosphatase 1 (DUSP1) also known as MAP kinase phosphatase 1 (MKP1) has specificity towards p44/42 MAPK, p38 MAPK and c-Jun-NH_2-terminal kinase (JNK). DUSP1 is over-expressed in a large proportion of primary breast cancer samples (27) and ectopic expression of DUSP1 inhibits doxorubicin induced apoptosis in mammary epithelial cells, as well as breast cancer cell lines (28).

Many chemotherapeutic agents induce apoptosis in part by activation of the JNK pathway (29), and both JNK cascade as well as cell death were functions probably regulated upon neoadjuvant treatment of breast tumors. As DUSP1 may limit JNK activity and cell death, DUSP1 higher expression in post-chemotherapy samples may have contributed to diminish chemotherapy effectiveness in these residual tumors.

In conclusion, we have evaluated residual samples from tumors considered partially responsive to AC regimen. Some of them retain their parental molecular signature whereas others present significant changes in their gene expression profile. Some genes are differentially expressed between pre- and post-chemotherapy samples, probably due to clone selection or drug induction, among them, CTGF and DUSP1, which induction in residual samples may be a reflection of resistance to further administration of AC regimen.

Acknowledgments

The authors would like to acknowledge the helpful assistance of Dr Olavo Feher and Dr José Getúlio Martins Segalla for patients accrual and treatment, Dr Antônio Hugo J.F.M. Campos, for sample processing, Mrs. Waleska Martins for technical support, Mrs. Maria José Gonçalves Benevides for secretarial help and Mrs. Cristina Piñeiro Grandal for figure edition. This work was supported by FAPESP, CNPQ and CAPES.

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