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Gene Expression Profiles Discriminate between Pathological Complete Response and Resistance to Neoadjuvant FEC100 in Breast Cancer

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Abstract. *Background: In breast cancer treatment, FEC100 (fluorouracil, epirubicin and cyclophosphamide) chemotherapy delivered in a neoadjuvant setting is still applied empirically to all patients. The aim of this study was to establish a multigene classifier of sensitivity to neoadjuvant FEC100. Materials and Methods: cDNA nylon microarrays, containing 15,000 genes, were used to analyze the gene expression profiles of tumour biopsies collected before chemotherapy: 8 were typed as pathological complete responders and 8 as non-responders according to their histological and clinical responses. Results: A classifier was generated by means of Linear Discriminant Analysis and was evaluated by leave-one-out cross-validation. The difference of expression of the NDUFB5 gene (NADH dehydrogenase 1 beta subcomplex, 5), the best discriminating gene, was verified using RT-PCR. Conclusion: This preliminary work requires further investigations, especially in terms of larger cohorts, before the results can be transferred to clinical practice.*

Neoadjuvant chemotherapy is the treatment of choice for patients with inflammatory breast cancer (IBC), locally advanced breast cancer (LABC) or to allow breast-conserving surgery (1). Pathological complete response (pCR) (absence of any tumour [invasive or *in situ*] and no lymph node involvement on pathological examination) after neoadjuvant therapy is demonstrated in less than 4-34% of women. The neoadjuvant setting provides an *in vivo* assessment of tumour response to the particular drug regimen and, hence, a unique opportunity to identify molecular predictors of response to chemotherapeutic agents; the corresponding findings are more accurate than those derived from experiments with tumour cells *in vitro* or tumour xenografts *in vivo* and, probably, are more easily adaptable to clinical practice.

Thus, in order to decrease breast cancer mortality, one urgent need is to discover markers providing information about chemotherapy sensitivity, because delivering a first-line chemotherapy to a non-responder (NR) patient leads to loss of time, collateral toxicity and may induce cross-resistance at second-line chemotherapy (2). Currently, the FEC100 combination (5-fluorouracil [5-FU] 500 mg/m^2, epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2) is the leading therapy in neoadjuvant and adjuvant breast cancer treatment (3).

The development of cDNA microarray technology has facilitated the analysis of genome-wide expression profiles that could identify genes related to responsiveness to chemotherapy. Most reports investigated the resistance of different cell lines to chemotherapeutic agents, but did not take into account *in vivo* microenvironment-dependent resistance mechanisms (4-6). Recently, research focusing on various combinations of chemotherapeutic agents have been published, but not on the FEC100 combination (7-10), which is still applied empirically to all patients.

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Table I. *Clinicopathological characteristics of the 16 patients studied.*

Patient no.	Response*	Age at diagnosis	TNM stage	Histological type	EE	OR	PR	ErbB-2	Neoadjuvant chemotherapy	Nodal status*	Histological residue*		Follow-up data	
											type	size (mm)	months	status
1	NR	49	T2N1M0	IDC	II	+	+	2+	6FEC100	1/7	IDC	28	38	DF
2	NR	61	T4N0M0	IDC	II	-	-	0	4FEC100+3D	7/7	IDC	80	9	dead
3	NR	56	T2N0M0	IDC	I	+	+	0	6FEC100	0/6	IDC	13	30	DF
4	NR	54	T2N1M0	IDC	II/III	+	+	2+	6FEC100	0/9	IDC	40	50	DF
5	NR	49	T2N1M0	IDC	*nd*	+	*nd*	*nd*	6FEC100	1/12	IDC	40	65	DF
6	NR	38	T2N0M0	IDC	II	+	+	2/3+	4FEC100	1/9	IDC	65	41	DF
7	NR	56	T3N2M0	IDC	II	-	-	0	4FEC100+3D	*nd*	*nd*	*nd*	5	dead
8	NR	32	T3N1M0	IDC	II	-	-	0	5FEC100+2D/C+4D	5/5	IDC	13	13	dead
9	pCR	46	T2N0M0	IDC	III	-	-	nd	6FEC100	0/7	-	0	73	DF
10	pCR	34	T2N1M0	IDC	II	+	+	3+	6FEC100	0/6	-	0	42	DF
11	pCR	66	T2N1M0	IDC	III	+	+	3+	6FEC100	0/6	-	0	10	DF
12	pCR	48	T3N1M0	IDC	II	+	+	0	6FEC100	0/5	-	0	58	DF
13	pCR	48	T2N0M0	IDC	II	-	-	1/2+	6FEC100	0/9	-	0	34	DF
14	pCR	54	T2N0M0	IDC	III	-	-	0	6FEC100	0/7	-	0	25	DF
15	pCR	43	T2N0M0	IDC	III	-	-	0	6FEC100	0/8	-	0	35	DF
16	pCR	61	T3N0M0	IDC	III	-	-	0	6FEC100	0/11	-	0	15	DF

* after neoadjuvant chemotherapy; NR: non-responder; pCR: pathological complete response.
nd: not determined.
EE: Elston Ellis histological grade.
OR: oestrogen receptor status.
PR: progesterone receptor status.
ErbB-2: immunohistochemistry grading of ErbB-2 expression (0 for no expression, 3+ for highest expression).
D: Docetaxel (Taxotere®).
C: Capecitabine (Xeloda®).
Nodal status: first number for positive nodes; second number for total investigated nodes.
DF: disease-free.

The current study used cDNA nylon microarray screening to identify predictive markers for FEC100. The cohort comprised 16 patients: 8 responders and 8 non-responders. Gene expression profiling was performed using cDNA microarrays containing 15,000 genes. A 14-gene classifier (*NDUFB5, SLC1A3, RAD54L, WBSCR27, WDR9, METTL2...*) was generated and showed a strong classifying power. The best discriminating gene was the NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16 kDA (*NDUFB5*), the difference in expression of which was validated by RT-PCR analysis.

Materials and Methods

Patients. The 16 patient cohort had been admitted to the Rene Gauducheau Cancer Centre, France. Informed consent was obtained from the patients to use their surgical specimens and clinicopathological data for research purposes, as required by the French Committee for the Protection of Human Subjects. The 16 eligible breast cancer biopsies were selected out of a total of 501 biopsies stored in our breast cancer tissue collection. The following inclusion criteria were strictly applied. After diagnostic biopsy, all the patients were initially treated with 6 cycles of neoadjuvant FEC100 chemotherapy, or less due to progressive disease (n=75). All biopsies presented tumour cells typed as infiltrating ductal carcinoma (IDC). No patient showed evidence of distant metastasis at the time of diagnosis. In order to more accurately identify the discriminating genes, patients who demonstrated a pCR (n=8; 10.6%) and NR patients (n=8) were exclusively selected; no partial response was used in the predictive analysis. The histological response was validated by a pathologist as complete if all evidence of primary carcinoma of the breast and node involvement disappeared after neoadjuvant FEC100 chemotherapy. Patients with either no or few clinical modifications of the tumoral appearance and with an important histological residue were classified as NR (diminution of tumour size <25%). In the latter group, the neoadjuvant FEC100 regimen was changed (n=3) or stopped (n=1) for 4 patients presenting progressive disease. The clinical characteristics of the patients are listed in Table I. No significant difference was found between the 2 groups for any of the clinical or biological characteristics (age, TNM, hormonal receptors, ErbB-2), with the exception of the Elston Ellis grading, which was more elevated in the pCR group (p=0.03).

Tissue samples. All patients underwent 2 biopsies before treatment. One biopsy was fixed in 10% neutral buffered formalin for standard histological analysis and immunohistochemistry to confirm the diagnosis. The second biopsy was frozen and conserved in liquid nitrogen until RNA extraction. Impression smears of all

biopsies were evaluated by a pathologist; only samples presenting tumour cells were eligible for the study.

RNA extraction and amplification. RNA was extracted from frozen core biopsies using the RNABle (Eurobio, les Ulis, France) protocol. The quality and the quantity of RNA samples were evaluated using the Agilent 2100 Bioanalyser RNA LabChip kit (Agilent Technologies, Palo Alto, CA, USA). To obtain a sufficient quantity of sample for microarray technology, 600 ng of total RNA were amplified using the Message Amp kit (Ambion, Austin, TX, USA), based on T7 polymerase transcription.

Nylon microarray technology. Nylon microarrays manufactured in our laboratory were used, which contained 15,000 cDNA clones chosen using the expressed sequence tag (EST) database from the NCBI [http://www.ncbi.nlm.nih.gov]. The same microarray tools were used and described in a previous work (11).

RNA labelling and microarray hybridization. One μg of antisense RNA was labelled by reverse transcription and incorporation of [α-33P]dCTP; then the nylon microarrays were hybridized with the labelled probes, as previously described (11).

Data acquisition and pre-processing. The DNA microarrays were scanned at 25-μm resolution using a Fuji BAS 5000 image plate system (Raytest, Paris, France). The hybridization signals were quantified using ArrayGauge software v.1.3 (Fuji Ltd, Tokyo, Japan). For each membrane, the data were normalized by the global intensity of hybridization.

Data processing and analysis. In order to detect the genes that were differentially-expressed between the 2 groups (pCR and NR), a Mann-Whitney *U*-test was performed (12). To avoid overfitting, a resampling technique was used (13). Genes with $p<0.01$ were kept for the following analysis.

Principal component analysis (PCA) was used both to visualise the data (*i.e.,* the expression of the discriminant genes) and as a precursory step to the hierarchical clustering for the descriptive study, on the one hand, and, on the other, for the discriminant analysis for the predictive study.

Hierarchical clustering was performed on the first principal components to evaluate similarities and dissimilarities of patient profiles (14). The Ward algorithm was employed.

Linear Discriminant Analysis (LDA) was performed to generate a classifier of the histological response to the treatment. The leave-one-out cross-validation (LOOCV) method was used to assess the performance of the classifier and, again, provide protection against overfitting.

Validation by RT-PCR analysis. The difference in expression of the *NDUFB5* gene between the pCR and NR groups was evaluated using RT-PCR, to confirm the reliability of our approach. One μg of amplified RNA was used as the template in a total volume of 20 μL reverse transcription reaction system, using Superscript II (Invitrogen, Carlsbad, CA, USA) primed with random hexamers. The cDNA were then amplified by PCR using the primers: sense, 5'-tgaggagtttggcttccagt-3' and antisense, 5'-cctgagttgcccctcaataa-3'. The 229 bp bands were visualised by ethidium bromide staining of the gel and estimated relative to the DNA ladder. After densitometry scanning, the *NDUFB5* levels were measured.

Results

Data processing and analysis. After data pre-processing, 10,694 genes were taken into account for statistical analysis.

Univariate analysis. To identify a subgroup of genes likely to discriminate NR patients from patients with a pCR, the Mann-Whitney *U*-test was applied in association with a technique for resampling to generate robust estimates. Fourteen genes were found discriminant at $p<0.01$ (Table II).

Descriptive analysis. To reduce the data dimension and to precisely study the links between the selected genes, and the similarities or disparities of the patients' profiles, a PCA was performed on the 14 genes with a p value < 0.01, based on the 16 patients. The NR patients formed a very compact group; on the contrary, the patients with a pCR were more heterogeneous (Figure 1). To visualise distances between patients, hierarchical clustering was applied on the first principal components. On the dendrogram, the 2 groups appear very far apart; the homogeneity of the NR group and the heterogeneous structure of the pCR group are also shown (Figure 1).

Predictive analysis. To build the classifier, PCA was performed to reduce the dimension of the data. The classifier was then built by means of LDA. LOOCV was used to estimate the classification error rate. Each of the 16 patients was correctly classified by the classifier built from the other 15.

Validation by RT-PCR analysis. Using RT-PCR, the expression of the *NDUFB5* gene was compared (Figure 2a). Figure 2b shows that the *NDUFB5* expression of NR samples was globally down-regulated in comparison with the expression of the pCR samples, which is in concordance with the microarray results.

Discussion

A classifier was built, composed of 14 genes, for tumour responsiveness to neoadjuvant FEC100 chemotherapy. Gene ontology annotations of these genes showed some similarities throughout the molecular function, biological process or cellular component (Table II).

Attention was focused on *NDUFB5*, the best discriminating gene, the difference of expression of which between the pCR and NR groups was confirmed using RT-PCR. *NDUFB5* is a subunit of Complex I (aka, NADH:ubiquinone oxidoreductase) which belongs to the electron transport chain, including 2 mobile electron carriers and 5 multiprotein complexes designated as Complexes I – V (15). The 5 multiprotein assemblies

Table II. *Gene ontology annotations and statistic characteristics of the top 14 genes.*

Rank	Resampled *p* value	Symbol	Gene ontology		
			Molecular function	Biological process	Cellular component
1	0.0022	*NDUFB5*	NADH dehydrogenase activity Oxidoreductase activity	Mitochondrial electron transport, NADH to ubiquinone	Mitochondrion
2	0.0028	*SLC1A3*	Sodium:dicarboxylate symporter activity Symporter activity L-glutamate transporter activity	Neurotransmitter uptake Synaptic transmission Transport Dicarboxylic acid transport	Integral to membrane Membrane fraction
3	0.0034	*RAD54L*	DNA binding Helicase activity Hydrolase activity ATP binding	DNA recombination DNA repair Cell growth and/ or maintenance Meiosis	Nucleus
4	0.0037	*WBSCR27*	Unknown	Unknown	Unknown
5	0.0039	*WDR9*	Transcription regulator activity	Protein binding	Nucleus
6	0.0054	*METTL2*	Transferase activity SAM-dependent methyltransferase activity		
7	0.0057	*MRPS10*	Structural constituent of ribosome	Protein biosynthesis	Ribosome Intracellular Mitochondrial ribosome Mitochondrion
8	0.0075	*CTSS*	Hydrolase activity Cathepsin S activity	Immune response Proteolysis and peptidolysis	Extracellular region Lysosome
9	0.0078	*PCGF6*	Zinc ion binding DNA binding Ubiquitin-protein ligase activity	Protein ubiquitination	Ubiquitin ligase complex
10	0.0079	*GHITM*			Integral to membrane
11	0.0093	*PIM2*	Protein serine/threonine kinase activity Transferase activity ATP binding	Protein amino acid phosphorylation	
12	0.0093	*PCTP*	Lipid binding Phosphatidylcholine transporter	Lipid transport	Cytosol
13	0.0094	*RNF13*	Ubiquitin-protein ligase activity Zinc ion binding Peptidase activity	Protein ubiquitination Proteolysis and peptidolysis	Ubiquitin ligase complex Nucleus
14	0.0095	*KNSL7*	DNA binding Motor activity ATP binding	Cell proliferation Mitosis	Centrosome Microtubule-associated complex Plus-end kinesin complex

consist of 4 to 46 (Complex I) different subunits. These components act in a complicated biological process, consisting of a cascade of enzymic events involved in the production of ATP, the consequent generation of a proton gradient across the mitochondrial inner membrane and, eventually, the conversion of ADP to ATP. The overall function of Complex I is to pass electrons from NADH to ubiquinone, while pumping hydrogen ions out of the mitochondrial matrix into the inter-membrane space.

Anthracyclines are known for their complex cytotoxic mechanisms involving inhibition of topoisomerase II, intercalation into DNA, generation of reactive oxygen species (ROS) and induction of apoptosis (16). A relationship between resistance to anthracyclines and NADH dehydrogenase have been shown in a few studies. Davies *et al.* demonstrated that Complex I is the mitochondrial site of anthracycline reduction, showing that the generation of mitochondrial ROS accounts, in part, for the cytotoxic effects of the anthracyclines (17). Wong *et al.* found a down-

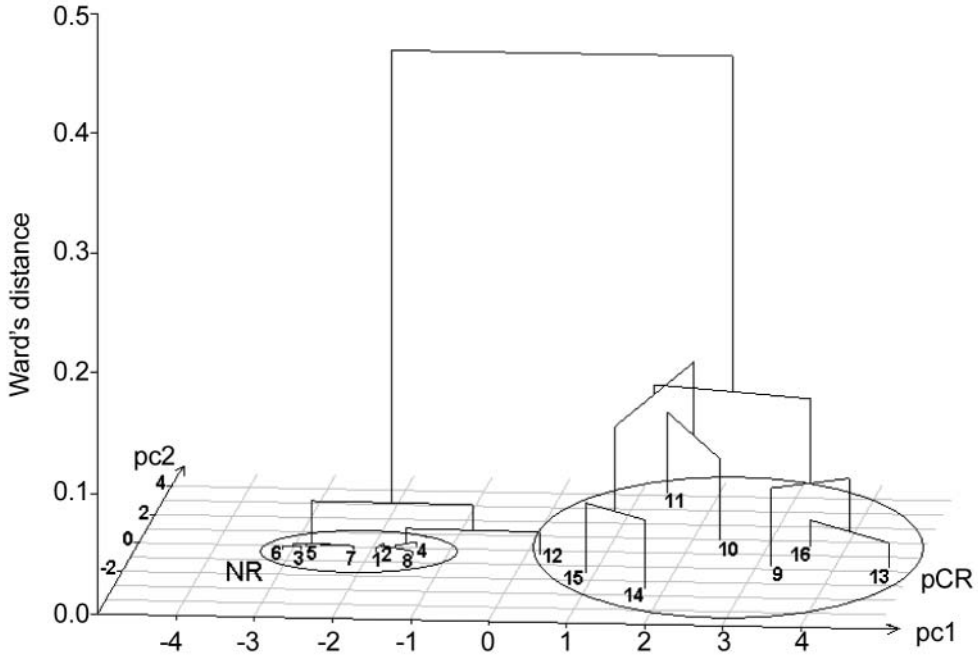
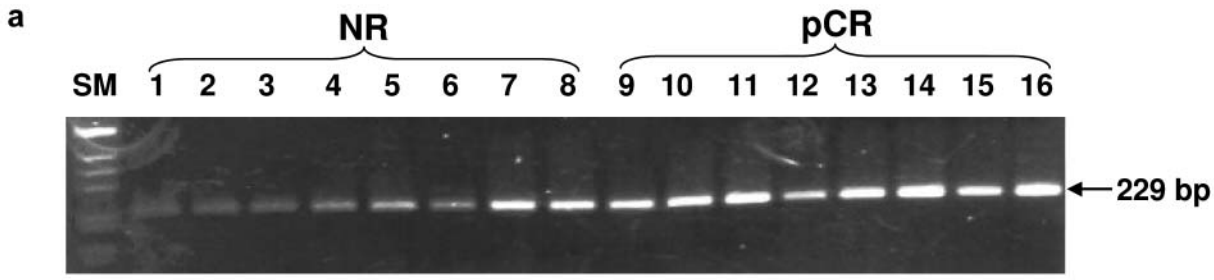


Figure 1. *Projection of the 8 NR patients and 8 pCR patients onto the first principal plan. The x-axis (pc1) represents the first principal component and the y-axis (pc2) the second one. The 8 NR patients are numbered from 1 to 8 and the 8 pCR patients are numbered from 9 to 16. The dendrogram displays the results from the hierarchical clustering based on the first 5 principal components.*



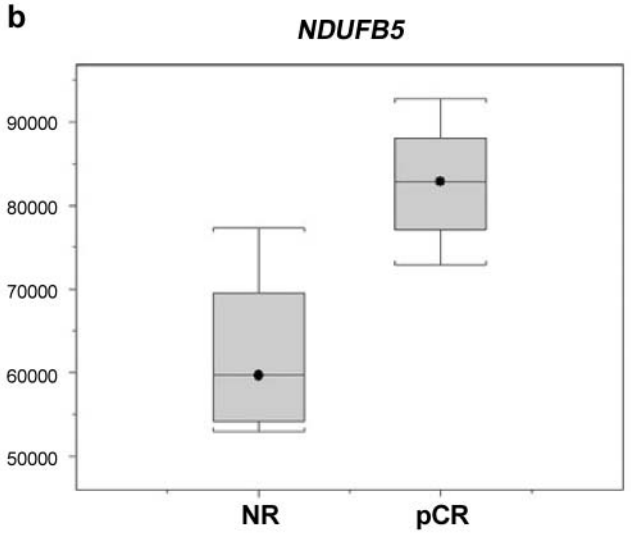


Figure 2. *Confirmation of cDNA microarray results by RT-PCR analysis. (2a) Two % agarose gel electrophoresis of 8 NR and 8 pCR samples amplified by RT-PCR, as described in Materials and Methods (SM: HaeIII-digested pBR322 size marker). (2b) The box-plots show the distribution of NDUFB5 expression in the NR and pCR samples.*

regulation of the mitochondrial NADH dehydrogenase III (ND3) gene in the doxorubicin-resistant A431 cell line derived from human squamous carcinoma (18). They suggested that the level of ND3 may determine doxorubicin-induced apoptosis in cells *via* its influence on drug-induced ROS formation and that the resistance to doxorubicin-induced apoptosis is probably due to a reduction in doxorubicin-induced ROS formation. The importance of ROS in doxorubicin-induced apoptosis was confirmed by the fact that the presence of catalase, a free radical scavenger, could suppress doxorubicin-induced ROS formation and subsequently drug-induced apoptosis. The presumed relationship between ROS and apoptosis was strengthened by Li *et al.*, who demonstrated that in isolated HL-60 cells rotenone-induced mitochondrial ROS production was closely related to rotenone-induced apoptosis (19). The results from manganese superoxide dismutase-overexpressing HT1080 cells confirmed the conclusion that rotenone induced apoptosis *via* an induction of mitochondrial ROS production. In 11 human cancer cell lines, *NDUFB6* gene expression was shown to have a negative correlation with sensitivity to epirubicin (20). Later, down-regulation of NADH dehydrogenase Fe-S protein 6 (*NDUFS6*) was observed in the doxorubicin-resistant K562 cell line derived from human leukaemia (21). In the same year, a mild Complex I deficiency and resistance to doxorubicin was shown in M010b glioma cells (22). Our results, which demonstrated a FEC100-predictive role for *NDUFB5* and its down-regulation in FEC100-resistant breast tumours, are in concordance with the aforementioned researches. Furthermore, another gene included in the 14-gene classifier, *MRPS10*, underlines the discriminating role of mitochondrial metabolism in tumour responsiveness. In the present study, the mechanism of resistance to FEC100 could be strongly linked to resistance to epirubicin. Anthracycline-based resistance mechanisms could be linked to the down-regulation of different subunit genes of the NADH dehydrogenase Complex I and, hence, of the Complex I function, ROS modulation and mitochondria-induced apoptosis.

In conclusion, based on an *in vivo* 15,000 gene profiling of 2 perfectly annotated and distinct, but limited, patient groups (pCR and NR), interesting preliminary results were obtained. As underlined by previous studies, some of the genes retained in the 14-gene FEC100 classifier are clearly linked to the mechanisms of sensitivity or resistance to chemotherapy, or to breast cancer prognosis and development (*NDUFB5, PIM2, KNSL7, RAD54L*). Electron transport chain, hypoxia and cell proliferation are indicated as being key mechanisms responsible for cell responsiveness to chemotherapy. With these encouraging results, the practical value of this classifier remains to be validated in sequential series of clinical studies analogous to phase II and III clinical trials.

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