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Ivan Bièche[1,2], Igor Girault[1], Estelle Urbain[1], Sengül Tozlu[1] and Rosette Lidereau[1]

[1]Laboratoire d'Oncogénétique – INSERM E0017, Centre René Huguenin, St-Cloud, France
[2]Laboratoire de Génétique Moléculaire – UPRES EA 3618, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes – Paris V, Paris, France

Corresponding author: Ivan Bièche (e-mail: i.bieche@stcloud-huguenin.org)

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Abstract

Introduction: Little is known of the function and clinical significance of intratumoral dysregulation of xenobiotic-metabolizing enzyme expression in breast cancer. One molecular mechanism proposed to explain tamoxifen resistance is altered tamoxifen metabolism and bioavailability.

Methods: To test this hypothesis, we used real-time quantitative RT-PCR to quantify the mRNA expression of a large panel of genes coding for the major xenobiotic-metabolizing enzymes (12 phase I enzymes, 12 phase II enzymes and three members of the ABC transporter family) in a small series of normal breast (and liver) tissues, and in estrogen receptor alpha (ERα)-negative and ERα-positive breast tumors. Relevant genes were further investigated in a well-defined cohort of 97 ERα-positive postmenopausal breast cancer patients treated with primary surgery followed by adjuvant tamoxifen alone.

Results: Seven of the 27 genes showed very weak or undetectable expression in both normal and tumoral breast tissues. Among the 20 remaining genes, seven genes (*CYP2A6*, *CYP2B6*, *FMO5*, *NAT1*, *SULT2B1*, *GSTM3* and *ABCC11*) showed significantly higher mRNA levels in ERα-positive breast tumors than in normal breast tissue, or showed higher mRNA levels in ERα-positive breast tumors than in ERα-negative breast tumors.

In the 97 ERα-positive breast tumor series, most alterations of these seven genes corresponded to upregulations as compared with normal breast tissue, with an incidence ranging from 25% (*CYP2A6*) to 79% (*NAT1*). Downregulation was rare. *CYP2A6*, *CYP2B6*, *FMO5* and *NAT1* emerged as new putative ERα-responsive genes in human breast cancer. Relapse-free survival was longer among patients with *FMO5*-overexpressing tumors or *NAT1*-overexpressing tumors ($P = 0.0066$ and $P = 0.000052$, respectively), but only *NAT1* status retained prognostic significance in Cox multivariate regression analysis ($P = 0.0013$).

Conclusions: Taken together, these data point to a role of genes coding for xenobiotic-metabolizing enzymes in breast tumorigenesis, *NAT1* being an attractive candidate molecular predictor of antiestrogen responsiveness.

Keywords: breast cancer, prognostic value, real-time RT-PCR quantification, tamoxifen xenobiotic-metabolizing enzyme expression

Introduction

Breast cancer growth is regulated by estrogen, which acts by binding to its estrogen receptor alpha (ERα). The presence of ERα in breast tumors is used as a biological marker to identify patients who may respond to endocrine agents such as tamoxifen. However, one-half of the patients with ERα-positive tumors fail to respond favorably to antiestrogen treatment [1,2].

ABCC1 = ATP binding cassette C1 isoform; AUC = area under the curve; *CYP2A6* = cytochrome P450 2A6 isoform; *CYP2B6* = cytochrome P450 2B6 isoform; ERα = estrogen receptor alpha; *ESR1*/ERα = estrogen receptor alpha; *FMO5* = flavin-containing monooxygenase 5 isoform; *GSTM3* = glutathione S-transferase M3 isoform; *MKI67* = proliferation-related Ki-67 antigen; *NAT1* = *N*-acetyltransferase 1 isoform; PCR = polymerase chain reaction; ROC = receiver–operating characteristic; RT = reverse transcriptase; *SULT2B1* = sulfotransferase 2B1 isoform; *TBP* = TATA box-binding protein.

Several mechanisms have been forwarded to explain this lack of response in ERα-positive patients, one being based on altered tamoxifen metabolism or bioavailability [3–5].

Tamoxifen is metabolized by phase I enzymes such as cytochromes P450, lactoperoxidase, microsomal epoxide hydrolase and flavin-containing monooxygenase [6–9]. Tamoxifen metabolites may have not only antiestrogenic activity, but also estrogenic or genotoxic actions [10–13]. These tamoxifen metabolites are secondarily detoxified by phase II enzymes (conjugation enzymes) such as catechol-*O*-methyltransferase, UDP-glucuronosyltransferases, glutathione S-transferases, sulfotransferases, *N*-acetyltransferases and NAD(P):quinone oxidoreductase [14–18].

The three main tamoxifen metabolites are tamoxifen-*N*-oxide (catalyzed by flavin-containing monooxygenase, *FMO1* and *FMO5*), 4-hydroxy-tamoxifen and *N*-desmethyl-tamoxifen (catalyzed by *CYP2B6*, *CYP2C9*, *CYP2D6*, *CYP2E1*, *CYP3A4*, etc. [7,8]). 4-Hydroxy-tamoxifen has the strongest antiestrogen activity (100-fold higher than tamoxifen itself) [6]. All three metabolites are secondarily detoxified by phase II enzymes [14–18].

Most xenobiotic-metabolizing enzymes are expressed in the liver, but some are also expressed in breast tissue. Intratumoral tamoxifen or metabolites (generated by hepatic metabolism) could thus undergo further transformation in the breast *in situ* [19]. Altered intratumoral expression of genes coding for xenobiotic-metabolizing enzymes is one potential mechanism of tamoxifen resistance.

Little is known of the function and clinical significance of the altered intratumoral expression of xenobiotic-metabolizing enzymes with respect to tamoxifen resistance. Lower tumor tamoxifen concentrations have been observed in tamoxifen-resistant tumors from breast cancer patients [20]. *CYP1A1* and *CYP1B1* expression is increased in antiestrogen-resistant human breast cancer cell lines [21]. Fritz and colleagues [22] recently identified microsomal epoxide hydrolase as a predictor of the tamoxifen response in breast cancer.

To further investigate the possible relationship between altered intratumoral expression of xenobiotic-metabolizing enzymes and both breast tumorigenesis and tamoxifen resistance, we used real-time quantitative RT-PCR assays to quantify mRNA expression of a large panel of genes coding for the major xenobiotic-metabolizing enzymes (12 phase I enzymes, 12 phase II enzymes and three members of the ABC transporter family involved in multidrug resistance) in a small series of ERα-negative and ERα-positive breast tumors. Seven relevant genes thus identified were further investigated in a well-defined cohort of 97 ERα-positive postmenopausal breast cancer patients treated with primary surgery followed by adjuvant tamoxifen alone.

Materials and methods

Patients and samples

We analyzed tissue samples from primary breast tumors excised from 97 women at Centre René Huguenin from 1980 to 1994. The tumor samples were stored in liquid nitrogen immediately following surgery until RNA extraction. The patients (mean age, 71.1 years; range, 54–86 years) met the following criteria: primary unilateral nonmetastatic postmenopausal breast carcinoma; ERα-positive as determined at the protein level by biochemical methods (Dextran-coated charcoal method until 1988 and enzyme immunoassay thereafter) and at the mRNA level by *ESR1*/ERα real-time quantitative RT-PCR assay [23]; complete histological and biological information available from the primary tumors; no radiotherapy or chemotherapy before surgery; and clinical follow-up at Centre René Huguenin.

The standard prognostic factors are presented in Table 1. Thirty-one patients (32.0%) had modified radical mastectomy and 66 patients (68.0%) had breast-conserving surgery plus locoregional radiotherapy. Patients underwent physical examinations and routine chest radiography every 3 months for 2 years, and then annually. Mammograms were performed annually. The median follow-up was 6.5 years (range, 1.5–17.7 years). All the patients received postoperative adjuvant endocrine therapy (20 mg tamoxifen daily for 3–5 years), and no other treatment. Thirty-two patients relapsed (the distribution of first relapse events was 27 distant metastases, and five patients with both local and/or regional recurrences and metastases). Five ERα-negative tumors were also analyzed in order to investigate the relationship between target mRNA levels and ERα expression status.

Specimens of adjacent normal breast tissue from five breast cancer patients (patients who did not belong to the series of 97 patients analyzed in this study), and normal breast tissue from three women undergoing cosmetic breast surgery, were used as sources of normal breast RNA.

As xenobiotic-metabolizing enzymes are mainly expressed in the liver, we also analyzed a pool of mRNA from three normal human livers (Clontech, Palo Alto, CA, USA) in order to compare mRNA levels between normal breast and liver tissues.

Real-time RT-PCR

Theoretical basis

Quantitative values are obtained from the cycle number (Ct value) at which the increase in fluorescent signal associated with an exponential growth of PCR products starts to be detected by the laser detector of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). This was done using the PE Biosystems analysis software according to the manufacturer's manuals.

Table 1

Characteristics of the 97 postmenopausal patients with estrogen receptor alpha-positive breast tumors and relation to relapse-free survival

		Relapse-free survival	
	Number of patients	Relapses (%)	*P*[a]
Age			
≤ 70 years	47	18 (40.0)	NS (0.89)
> 70 years	50	14 (26.5)	
Histological grade[b,c]			
I + II	77	20 (26.0)	0.0057
III	19	11 (57.9)	
Lymph node status			
0	16	1 (6.2)	0.0018
1–3	52	15 (28.8)	
> 3	29	16 (55.2)	
Macroscopic tumor size[c]			
≤ 30 mm	66	18 (27.3)	0.028
> 30 mm	30	14 (46.7)	
Estrogen receptor alpha RNA status			
Low	33	16 (48.5)	NS (0.078)
Intermediate	32	6 (18.8)	
High	32	10 (31.2)	
Estrogen receptor beta RNA status			
Low	33	5 (15.1)	NS (0.062)
Intermediate	32	14 (43.8)	
High	32	13 (40.6)	

[a] *P* value, log-rank test. NS, not significant. [b] Scarff Bloom Richardson classification. [c] Information available for 96 patients.

The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (i.e. lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of the gene coding for the TATA box-binding protein (*TBP*) (a component of the DNA-binding protein complex TFIID) as the endogenous RNA control, and each sample was normalized on the basis of its *TBP* content.

Results, expressed as *n*-fold differences in target gene expression relative to the *TBP* gene (termed 'N*target*'), were determined by the formula: N*target* = $2^{\Delta Ct sample}$, where the ΔCt value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the *TBP* gene.

The N*target* values of the samples were subsequently normalized such that the mean of the N*target* values of the eight normal breast samples would equal a value of 1.

Target gene mRNA levels were confirmed using an additional endogenous RNA control for normalization; that is, the gene PPIA coding for the peptidylprolyl isomerase A (cyclophilin A).

Primers

Primers for the 27 xenobiotic-metabolizing target genes, the *ESR1*/ERα gene (coding for the ERα) and the *MKI67* gene (coding for the proliferation-related Ki-67 antigen) were chosen with the assistance of the computer program Oligo 5.0 (National Biosciences, Plymouth, MN, USA). We conducted BLASTN searches against 'dbEST', 'htgs' and 'nr' (the nonredundant set of the GenBank, EMBL and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequences chosen for the primers, and to confirm the absence of DNA polymorphisms. In particular, the primer pairs were selected to be unique when compared with the sequences of the closely related family member genes or of corresponding retropseudogenes. To avoid amplification of contaminating genomic DNA, one of the two primers was placed, if possible, in a different exon. For example, the upper primer of *TBP* was placed at the junction between exons 5 and 6, whereas the lower primer was placed in exon 6. In general, amplicons were between 70 and 120 nucleotides. Agarose gel electrophoresis allowed us to verify the specificity of PCR amplicons.

The 27 target genes tested in this study are presented in Table 2. The nucleotide sequences of the primers are available on request.

RNA extraction

Total RNA was extracted from breast specimens using the acid–phenol guanidinium method. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. The 18S and 28S RNA bands were visualized under ultraviolet light.

cDNA synthesis

RNA was reverse transcribed in a final volume of 20 µl containing 1 × RT buffer (500 µM each dNTP, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris–HCl; pH 8.3), 20 U RNasin Ribonuclease inhibitor (Promega, Madison, WI, USA), 10 mM dithiothreitol, 100 U Superscript II RNase H-reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA), 3 µM random hexamers (Pharmacia, Uppsala, Sweden) and 1 µg total RNA. The samples were incubated at 20°C for 10 min and at 42°C for 30 min, and RT was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min.

PCR amplification

All PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). PCR was performed using the SYBR® Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and at 65°C for 1 min.

Table 2

Target genes tested

Gene[a]	Genbank accession number	Chromosomal location	Description
Phase I enzymes			
CYP1A1	NM_000499	15q24.1	Cytochrome P450, subfamily IA, polypeptide 1
CYP1A2	NM_000761	15q24.2	Cytochrome P450, subfamily IA, polypeptide 2
CYP1B1	NM_000104	2p21	Cytochrome P450, subfamily IB, polypeptide 1
CYP2A6	NM_000762	19q13.2	Cytochrome P450, subfamily IIA, polypeptide 6
CYP2B6	NM_000767	19q13.2	Cytochrome P450, subfamily IIB, polypeptide 6
CYP2C9	NM_000771	10q24.1	Cytochrome P450, subfamily IIC, polypeptide 9
CYP2D6	NM_000106	22q13.1	Cytochrome P450, subfamily IID, polypeptide 6
CYP2E1	NM_000773	10q24.3-qter	Cytochrome P450, subfamily IIE, polypeptide 1
CYP3A4	NM_017460	7q22.1	Cytochrome P450, subfamily IIIA, polypeptide 4
FMO1	NM_002021	1q23-q25	Flavin-containing monooxygenase 1
FMO5	NM_018578	1q21	Flavin-containing monooxygenase 5
LPO	XM_042207	17q23.1	Lactoperoxydase
Phase II enzymes			
NQO1	NM_000903	16q22.1	NAD(P)H deshydrogenase, quinone 1
NAT1	NM_000662	8p23.1-p21.3	N-acetyltransferase 1
COMT	NM_000754	22q11.21	Catechol-*O*-methyltransferase
EPHX1	NM_000120	1q42.1	Epoxyde hydrolase 1, microsomal
SULT1A1	NM_001055	16p12.1	Sulfotransferase family, cytosolic, 1A, member 1
SULT2A1	NM_003167	19q13.3	Sulfotransferase family, cytosolic, 2A, member 1
SULT2B1	NM_004605	19q13.3	Sulfotransferase family, cytosolic, 2B, member 1
UGT1A1	NM_000463	2q37	UDP-glucuronosyltransferase, 1 family, polypeptide A1
GSTP1	NM_000852	11q13	Glutathion S-transferase pi 1
GSTM1	NM_000561	1p13.3	Glutathion S-transferase mu 1
GSTM3	NM_000849	1p13.3	Glutathion S-transferase mu 3
GSTT1	NM_000853	22q11.23	Glutathion S-transferase theta 1
Phase III proteins			
ABCB1	NM_000927	7q21.1	ATP-binding cassette, subfamily B (MDR/TAP), member 1 (MDR1)
ABCC1	NM_004996	16p13.1	ATP-binding cassette, subfamily C (CFTR/MRP), member 1 (MRP1)
ABCC11	NM_033151	16q12.1	ATP-binding cassette, subfamily C (CFTR/MRP), member 11 (MRP8)

[a] LocusLink symbol.

Statistical analysis

The distribution of mRNA levels was analyzed on the basis of their median values and ranges. Relationships between mRNA levels of the different target genes, and comparisons between median target gene mRNA levels and clinical, histological and biological parameters were based on nonparametric tests – namely the Mann–Whitney test (link between one qualitative parameter and one quantitative parameter) and the Spearman rank correlation test (link between two quantitative parameters). Differences between two populations were judged significant at confidence levels greater than 95% ($P < 0.05$).

To visualize the capacity of target gene mRNA levels to discriminate between patients who relapsed and those who did not relapse (in the absence of an arbitrary cutoff value), we used the receiver–operating characteristic

(ROC)–area under the curve (AUC) method [24]. When a molecular marker has no discriminatory value, the ROC curve lies close to the diagonal and the AUC value is close to 0.5. When a marker has strong discriminatory value, the ROC curve moves to the upper left-hand corner (or to the lower right-hand corner) and the AUC value is close to 1.0 (or to 0).

Relapse-free survival was determined as the interval between diagnosis and detection of the first relapse. Survival distributions were estimated by the Kaplan–Meier method [25], and the significance of differences between survival rates was ascertained using the log-rank test. Cox's proportional hazards regression model [26] was used to assess prognostic significance.

Results

mRNA expression of the 27 target genes in normal breast and liver tissue, and in ERα-negative and ERα-positive breast tumors

We analyzed the mRNA expression of 27 xenobiotic-metabolizing-enzyme genes, and the *MKI67* and *ESR1*/*ERα* genes, in a pool of normal liver tissue, in eight normal breast tissues, in five ERα-negative breast tumors and in 17 ERα-positive breast tumors.

Target gene mRNA levels were very low (detectable but not quantifiable by real-time quantitative RT-PCR assay, Ct > 35) in both normal and tumoral breast tissues for seven genes (*CYP1A1*, *CYP1A2*, *CYP2C9*, *CYP3A4*, *LPO*, *SULT2A1* and *UGT1A1*) out of the 27 xenobiotic-metabolizing enzyme genes. *CYP1A2*, *LPO* and *UGT1A1* were very weakly expressed (Ct > 35) in the pooled liver tissues, while the other four genes (*CYP1A1*, *CYP2C9*, *CYP3A4* and *SULT2A1*) showed significant expression (Ct < 30).

Means (± standard deviation) and ranges of mRNA levels for the 20 xenobiotic-metabolizing enzyme genes expressed in breast tissues, as well as for *ESR1*/ERα and for *MKI67*, are presented in Table 3. Target gene mRNA levels in the five ERα-negative breast tumors and in the 17 ERα-positive breast tumors (and in the pool of normal liver tissue) are expressed relative to the mean mRNA levels observed in the eight normal breast tissues.

From among the 20 xenobiotic-metabolizing enzyme genes, we selected seven genes of interest for further expression analysis in a large series of breast tumors (Table 3 and Fig. 1). These seven genes comprised four genes significantly upregulated in ERα-positive breast tumors as compared with normal breast tissue (i.e. *CYP2B6*, *NAT1*, *SULT2B1* and *ABCC11*), and three additional putative ER-responsive genes (*CYP2A6*, *FMO5* and *GSTM3*) that were significantly upregulated in ERα-positive tumors compared with ERα-negative tumors.

It is noteworthy (Table 3) that *CYP1B1* and *CYP2D6* were significantly upregulated in ERα-negative tumors compared with ERα-positive tumors, identifying them as candidate markers of tumor aggressiveness in ERα-negative human breast cancer.

Among the 20 xenobiotic-metabolizing enzyme genes, only *FMO5* showed markedly higher mRNA levels (>10-fold) in liver tissue than in breast tissue. *CYP1B1*, *NQO1* and *SULT2B1*, however, showed markedly lower mRNA levels (>10-fold) in liver tissue than in breast tissue. The other 16 genes showed close similar mRNA levels in the liver and the breast.

The mRNA levels of these 20 genes (except for *CYP2D6*; $r = +0.453$, $P = 0.033$, Spearman rank correlation test) were not associated with the *MKI67* mRNA level (a proliferation-related marker), suggesting that they are not upregulated in rapidly proliferating cells *in vivo* (data not shown).

GSTM1 and/or *GSTT1* mRNA was undetectable in some samples of both normal and tumoral breast tissue, probably owing to the particular polymorphism of these two genes (total absence of the two allele copies for these loci in 'allele null' patients).

The N*target* values (calculated as described in Materials and methods) presented in Table 3 are based on the amount of target messenger relative to the *TBP* endogenous control, in order to normalize the amount and quality of total RNA; similar results were obtained with a second endogenous RNA control (*PPIA*) coding for cyclophilin A (data not shown).

mRNA expression of seven selected genes in 97 ERα-positive breast tumors

We quantified mRNA levels of the *CYP2A6*, *CYP2B6*, *FMO5*, *NAT1*, *SULT2B1*, *GSTM3* and *ABCC11* genes in a well-defined cohort of 97 ERα-positive breast tumors from postmenopausal patients treated by surgery who only received tamoxifen hormonotherapy thereafter.

The ranges, means and medians of the mRNA levels of the seven target genes in this series of 97 breast tumors are summarized in Table 4. Major interindividual differences in mRNA levels (at least two orders of magnitude) were observed for all seven genes. For example, N_{CYP2B6} values ranged from 0.03 to 1053.1 (i.e. more than four orders of magnitude).

The cutoff points for altered gene expression in malignant breast tissues were determined using the N*target* values (calculated as described in Materials and methods) obtained for the eight normal breast RNA samples. The mean values for the eight normal breast N*target* plus five standard deviations were considered to represent the

Table 3

mRNA levels of *MKI67*, *ESR1*/ERα and the 20 target genes expressed in breast tissues

Gene	Normal liver tissue	Normal breast tissues, group I ($n = 8$)		ERα-negative breast tumors, group II ($n = 5$)		ERα-positive breast tumors, group III ($n = 17$)		Group III vs group I	Group III vs group II
		Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	P[a]	P[a]
CYP1B1	0.02	1.0[b] ± 0.3	0.5–1.3	11.2[b] ± 18.5	0.6–43.9	2.0[b] ± 2.8	0.3–11.8	NS	0.033
CYP2A6	9.8	1.0 ± 1.2	0.2–3.8	0.3 ± 0.1	0.1–0.4	737.9 ± 1931.2	0.01–7228.8	NS	0.048
CYP2B6	0.12	1.0 ± 0.8	0.1–2.3	0.2 ± 0.1	0.03–0.24	73.3 ± 87.7	0.8–249.8	0.0023	0.0090
CYP2D6	5.8	1.0 ± 0.6	0.3–1.7	3.4 ± 2.8	1.0–8.3	1.0 ± 0.6	0.3–2.3	NS	0.0054
CYP2E	0.97	1.0 ± 0.4	0.6–1.4	0.8 ± 0.5	0.2–1.4	1.5 ± 1.2	0.3–4.2	NS	NS
FMO1	4.7	1.0 ± 0.4	0.6–1.4	0.6 ± 0.5	0.2–1.3	0.6 ± 0.5	0.1–2.0	NS	NS
FMO5	28.5	1.0 ± 0.3	0.5–1.6	1.4 ± 2.1	0.2–5.1	3.4 ± 3.2	0.2–12.2	NS	0.042
NAT1	1.2	1.0 ± 0.3	0.5–1.5	0.5 ± 0.4	0.1–1.0	24.1 ± 29.0	1.2–105.2	0.0031	0.0009
COMT	0.47	1.0 ± 0.2	0.8–1.3	2.2 ± 1.5	0.3–4.3	0.9 ± 0.5	0.3–2.3	NS	NS
NQO1	0.009	1.0 ± 0.9	0.2–2.8	0.9 ± 0.9	0.1–2.0	1.4 ± 1.5	0.1–5.2	NS	NS
EPHX1	0.75	1.0 ± 0.9	0.2–2.1	0.3 ± 0.3	0.1–0.8	0.4 ± 0.3	0.1–1.0	NS	NS
SULT1A1	1.8	1.0 ± 0.7	0.3–1.6	0.6 ± 0.5	0.1–1.2	1.1 ± 0.9	0.4–3.3	NS	NS
SULT2B1	0.001	1.0 ± 1.0	0.0–2.9	1.7 ± 1.4	0.1–3.3	6.9 ± 6.9	0.4–24.0	0.0094	NS
GSTP1	0.54	1.0 ± 0.5	0.7–1.7	2.4 ± 4.0	0.2–9.4	0.9 ± 0.4	0.1–1.6	NS	NS
GSTM1	0.27	1.0 ± 1.2	0.0–2.6	0.05 ± 0.11	0.0–0.2	0.5 ± 1.1	0.0–4.3	NS	NS
GSTM3	0.23	1.0 ± 0.4	0.5–1.6	0.8 ± 1.3	0.1–3.0	2.7 ± 2.6	0.2–9.3	NS	0.011
GSTT1	0.35	1.0 ± 0.9	0.0–2.3	0.4 ± 0.3	0.1–0.7	0.6 ± 0.6	0.0–2.2	NS	NS
ABCB1	1.6	1.0 ± 1.2	0.4–2.8	0.3 ± 0.1	0.01–0.4	0.4 ± 0.4	0.1–1.3	NS	NS
ABCC1	0.16	1.0 ± 0.3	0.8–1.3	1.0 ± 0.7	0.3–2.2	0.9 ± 0.4	0.2–1.7	NS	NS
ABCC11	0.25	1.0 ± 0.9	0.4–3.3	31.2 ± 39.0	0.1–81.0	26.8 ± 36.8	0.1–108.3	0.039	NS
MKI67	70.3	1.0 ± 0.9	0.2–2.1	17.4 ± 6.6	10.0–26.6	13.0 ± 9.1	2.6–31.8	0.0023	NS
ESR1/ERα	0.002	1.0 ± 0.3	0.6–1.3	0.03 ± 0.02	0.01–0.05	29.6 ± 23.2	7.2–76.7	0.0023	0.0009

ERα, estrogen receptor alpha; SD, standard deviation. [a] P value, Mann–Whitney test. NS, not significant. [b] Mean of mRNA levels as determined in Materials and methods.

cutoff point for overexpression. The mean values for the eight normal breast N*target* minus two standard deviations (or N*target* value = 0.1 when the latter calculation gave a negative value) were considered to represent the cutoff point for underexpression. The percentage of tumors overexpressing and underexpressing the seven genes is presented in Table 4. It is noteworthy that most alterations corresponded to overexpression (from 25% of the tumors for *CYP2A6* to 79% for *NAT1*) and rarely to underexpression (from 0% for *NAT1* and *SULT2B1* to 15% for *CYP2A6*).

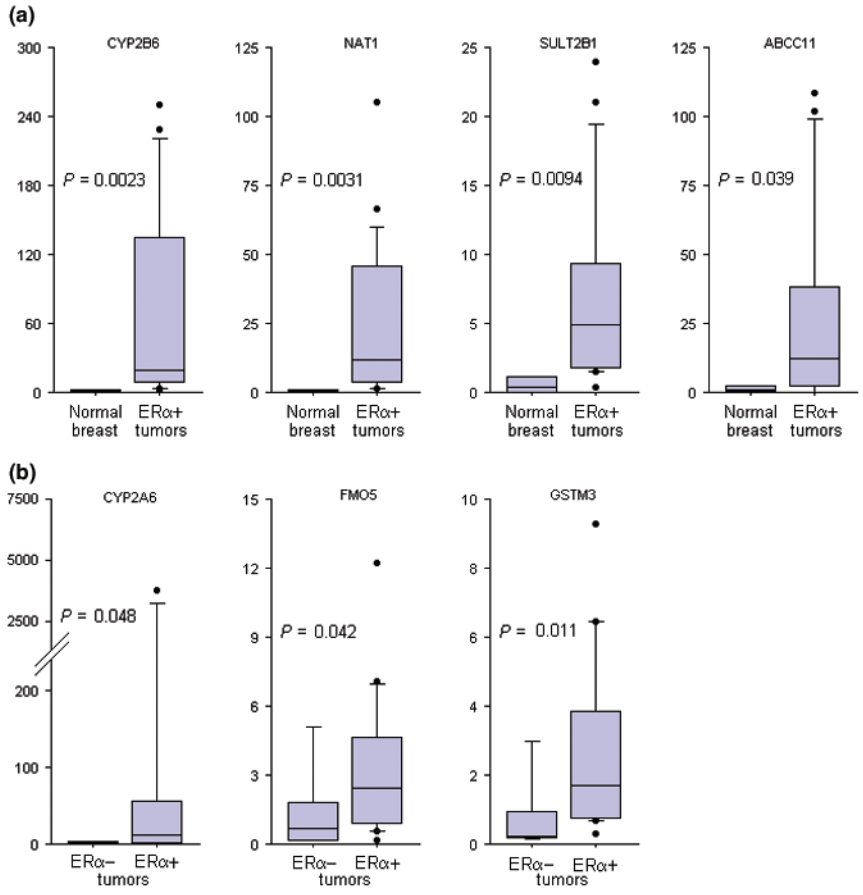
Relationships between mRNA values of the seven selected genes in 97 ERα-positive breast tumors

Using the Spearman rank correlation test (which compares continuous variables), we found a strong positive correlation between *CYP2A6*, *CYP2B6*, *FMO5* and *NAT1* mRNA levels (Table 5). We also quantified *ESR1*/ERα mRNA levels in this series of 97 ERα-positive breast tumors. We found a strong positive correlation *with CYP2A6*, *CYP2B6*, *FMO5* and *NAT1* mRNA levels and, to a lesser extent, with *GSTM3* mRNA levels.

Prognostic value of the seven selected genes in 97 ERα-positive breast tumors

The comparison of median mRNA levels in tumors from patients without relapse ($n = 65$) and in tumors from patients with relapse ($n = 32$) identified significant differences in the expression of three genes (*CYP2B6, FMO5* and *NAT1*) (Table 6). The three genes showed lower mRNA levels in the patients who relapsed than in those who did not relapse. The prognostic performance of each of the

Figure 1



mRNA expression levels of the seven selected genes. **(a)** Comparison between normal breast versus estrogen receptor alpha (ERα)-positive breast tumors. **(b)** Comparison between ERα-negative versus ERα-positive breast tumors. *P* value, Mann–Whitney test. Box central bar, median mRNA level.

seven selected genes for relapse was assessed using ROC curves. The overall prognostic value of these candidate molecular markers was compared using their AUC values, which identified *NAT1* (AUC–ROC, 0.24) as the most discriminatory gene (Table 6).

Univariate and multivariate prognostic analyses were then applied to *CYP2B6*, *FMO5* and *NAT1* status according to patient survival. As the percentage of patients with *CYP2B6*-overexpressing and *NAT1*-overexpressing tumors was high (76% and 79%, respectively; Table 4), the overexpressing tumors were subdivided into two equal subgroups with moderate and strong overexpression for univariate analysis (log-rank test). This analysis showed that longer relapse-free survival was linked to *NAT1* overexpression ($P = 0.000052$; Fig. 2a) and to *FMO5* overexpression ($P = 0.0066$; Fig. 2b). With regard to the two subgroups of *NAT1*-overexpressing tumors, the higher the *NAT1* mRNA level, the better the outcome (Fig. 2a).

Relapse-free survival was not significantly associated with *CYP2B6* mRNA status ($P = 0.078$; Fig. 2c).

Multivariate analysis (Cox proportional hazards model) was used to assess the influence of *NAT1* and *FMO5* mRNA status on relapse-free survival, together with classical prognostic parameters identified by univariate analysis (histopathological grade, lymph node status and macroscopic tumor size) in this same series of patients (Table 1). Only *NAT1* mRNA status and lymph node status retained their prognostic significance (Table 7; $P = 0.0013$ and $P = 0.016$, respectively).

Discussion

To test the hypothesis that altered tamoxifen metabolism and bioavailability could explain some cases of resistance, and to identify new candidate molecular markers to predict antiestrogen responsiveness in breast cancer, we used real-time quantitative RT-PCR to measure the

Table 4

mRNA levels of seven selected genes in 97 estrogen receptor alpha-positive breast tumors

	mRNA levels			Expression status		
Gene	Mean ± SD	Median	Range	Underexpressed[a]	Normal	Overexpressed[b]
CYP2A6	344.0[c] ± 1540.6	0.9	0.001–9741.1	15 (15.5)[d]	58 (59.8)[d]	24 (24.7)[d]
CYP2B6	103.3 ± 172.7	37.0	0.03–1053.1	1 (1.0)	22 (22.7)	74 (76.3)
FMO5	5.2 ± 6.3	2.6	0.10–30.1	1 (1.0)	43 (44.3)	53 (54.7)
GSTM3	3.1 ± 3.5	2.0	0.07–21.2	2 (2.1)	60 (61.8)	35 (36.1)
SULT2B1	7.5 ± 10.5	4.5	0.17–84.4	0	61 (62.9)	36 (37.1)
NAT1	46.5 ± 55.4	21.5	1.1–295.6	0	20 (20.6)	77 (79.4)
ABCC11	37.1 ± 60.4	15.9	0.04–461.7	3 (3.1)	27 (27.8)	67 (69.1)

[a] Less than mean values for the normal breast N*target* minus two standard deviations (SDs) (or N*target* value = 0.1 when the latter calculation gave a negative value). [b] Greater than mean values for the normal breast N*target* plus five SDs. [c] The *n*-fold differences in target gene expression relative to the TATA box-binding protein (*TBP*) gene and the normal breast tissues. [d] Number of patients (percentage).

Table 5

Relationships between mRNA values of the seven selected genes and the *ERα* gene in the 97 estrogen receptor alpha (ERα)-positive breast tumor series

	CYP2B6	*FMO5*	*NAT1*	*SULT2B1*	*GSTM3*	*ABCC11*	*ESR1*/ERα
CYP2A6	+ 0.471 [< 0.001]	+ 0.171 [NS (0.091)]	+ 0.248 [0.014]	+ 0.084 [NS (0.42)]	– 0.037 [NS (0.72)]	– 0.173 [NS (0.086)]	+ 0.281 [0.0053]
CYP2B6		+ 0.525 [< 0.001]	+ 0.433 [< 0.001]	+ 0.083 [NS (0.42)]	+ 0.022 [NS (0.83)]	+ 0.022 [NS (0.82)]	+ 0.409 [< 0.001]
FMO5			+ 0.420 [< 0.001]	– 0.099 [NS (0.34)]	– 0.008 [NS (0.93)]	+ 0.216 [0.032]	+ 0.316 [0.0018]
NAT1				+ 0.077 [NS (0.46)]	+ 0.104 [NS (0.31)]	+ 0.041 [NS (0.69)]	+ 0.293 [0.0037]
SULT2B1					+ 0.232 [0.021]	+ 0.049 [NS (0.64)]	+ 0.091 [NS (0.38)]
GSTM3						+ 0.198 [0.049]	+ 0.239 [0.018]
ABCC11							+ 0.023 [NS (0.82)]

Data presented as Spearman rank correlation coefficient [*P* value (Spearman rank correlation test)]. NS, not significant.

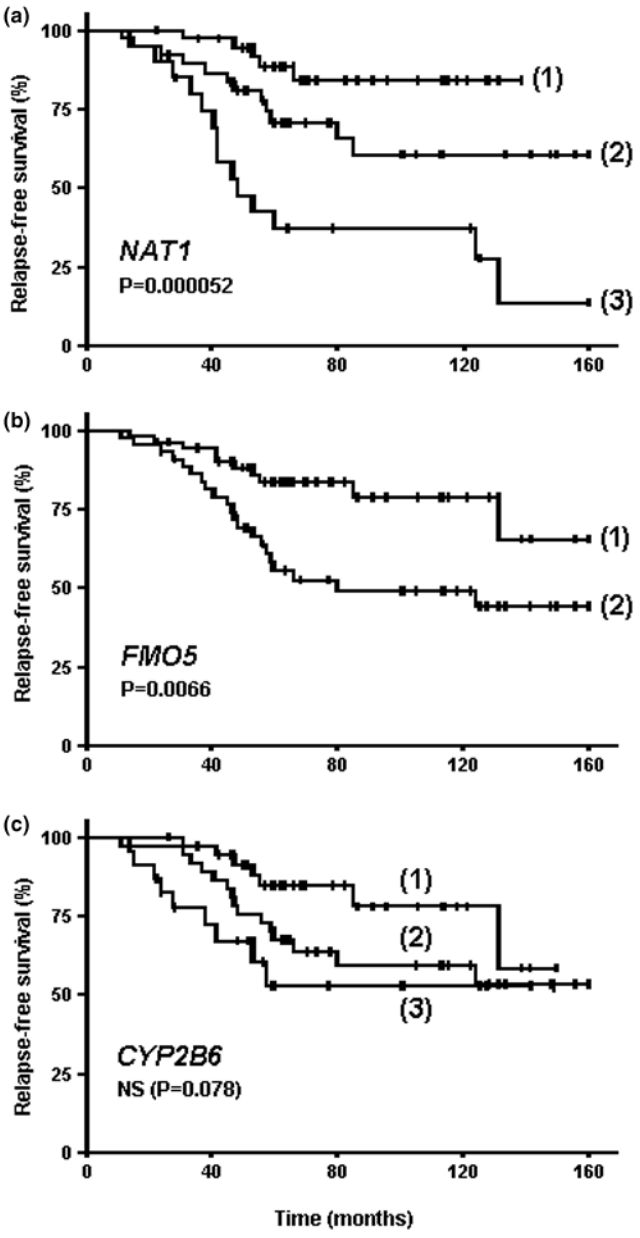
Table 6

Relationships between the prognostic (± relapses) and the mRNA levels of the seven selected genes in 97 estrogen receptor alpha-positive breast tumors

Gene	Tumors without relapses (*n* = 65)	Tumors with relapses (*n* = 32)	*P*[a]	ROC–AUC[b]
CYP2A6	1.2 (0.001–9741.1)[c]	0.8 (0.01–7228.8)	NS (0.17)	0.41 (0.29–0.54)[d]
CYP2B6	56.1 (0.3–1053.1)	14.7 (0.03–249.8)	0.011	0.34 (0.23–0.45)
FMO5	3.9 (0.2–30.1)	1.4 (0.1–23.9)	0.0016	0.30 (0.19–0.41)
GSTM3	2.1 (0.07–12.2)	1.7 (0.09–21.2)	NS (0.80)	0.48 (0.35–0.62)
SULT2B1	4.9 (0.2–84.4)	3.5 (0.4–21.0)	NS (0.65)	0.47 (0.35–0.59)
NAT1	35.9 (1.6–295.6)	10.0 (1.1–134.1)	0.000047	0.24 (0.14–0.35)
ABCC11	15.9 (0.06–195.5)	16.6 (0.04–461.7)	NS (0.60)	0.46 (0.33–0.60)

[a] *P* value, Mann–Whitney test; NS, not significant. [b] Receiver–operating characteristics (ROC)–area under curve (AUC) analysis. [c] Median (range) of gene mRNA levels. [d] AUC value (95% confidence interval).

Figure 2



Relapse-free survival curves. **(a)** Patients with strong *NAT1* overexpression (1), patients with moderate *NAT1* overexpression (2) and patients with normal *NAT1* expression (3). **(b)** Patients with *FMO5* overexpression (1) and patients with normal *FMO5* expression (2). **(c)** Patients with strong *CYP2B6* overexpression (1), patients with moderate *CYP2B6* overexpression (2) and patients with normal *CYP2B6* expression (3). NS, not significant.

Table 7

Multivariate analysis of relapse-free survival

	Relapse-free survival		
Variable	Regression coefficient	RR (95% CI)	*P*[a]
NAT1 status	−0.84		0.0013
Normal expression		1	
Moderate overexpression		0.43 (0.26-0.72)	
Strong overexpression		0.19 (0.07-0.52)	
Lymph node status	+0.77		0.016
0		1	
1–3		2.17 (1.15-4.09)	
> 3		4.72 (1.33-16.7)	

CI, confidence interval; RR, relative risk. [a] *P* value, Cox's proportional hazards regression model.

expression of a large panel of genes ($n = 27$) coding for major xenobiotic-metabolizing enzymes. These 27 genes encode 12 phase I enzymes (including *CYP2C9*, *CYP2D6*, *CYP3A4* and *FMO1*, known to be involved in the hepatic metabolism of tamoxifen [6,8]), 12 phase II enzymes and three members of the ABC transporter family involved in multidrug resistance in a series of human breast tumors.

Real-time quantitative RT-PCR has a major advantage over cDNA microarrays in the present setting in that it can distinguish closely related family member genes. Indeed, some xenobiotic-metabolizing enzyme genes are clustered in the same chromosomal region, and their nucleotide sequences show considerable homology. This is the case for the genes coding for certain cytochrome P450s, UDP-glucuronosyltransferases, glutation S-transferases and sulfotransferases. Real-time RT-PCR can use primer pairs that are unique relative to closely related family member genes. It is important to study these highly homologous genes individually, as they frequently code for enzymes with very different substrates.

Although we did not study all existing xenobiotic-metabolizing enzyme genes, our results nevertheless demonstrate the usefulness of real-time RT-PCR and identify several candidate marker genes of potential clinical value.

Xenobiotic-metabolizing enzyme genes in breast cancer have mainly been studied by investigating the relationship between genetic polymorphisms and cancer susceptibility [19]. This DNA-level approach was not suited to our aims, as it does not distinguish between hepatic gene expression and/or mammary gene expression.

We first quantified the mRNA expression of 27 genes coding for major xenobiotic-metabolizing enzymes in a small series of ERα-negative ($n = 5$) and ERα-positive ($n = 17$) breast tumors. Seven genes of interest were then further investigated in a well-defined cohort of 97 ERα-

positive postmenopausal breast cancer patients treated with primary surgery followed by adjuvant tamoxifen alone. This two-step strategy significantly limited the required number of PCR experiments.

The results of the first step yielded the following information about the involvement of xenobiotic-metabolizing enzymes in breast tumorigenesis. Among the 27 genes we identified seven genes (*CYP1A1*, *CYP1A2*, *CYP2C9*, *CYP3A4*, *LPO*, *SULT2A1* and *UGT1A*) whose expression is very weak or undetectable in breast tissue, in partial agreement with published results [27–29]. In particular, the recent study of Iscan and colleagues [30] observed marked expression of *CYP1B1*, *CYP2B6*, *CYP2D6* and *CYP2E1* in breast tumors, but low expression or no expression of *CYP1A1* and *CYP3A4*. The only discrepancy with our study concerns *CYP2A6* expression, which was not observed in breast tissue by Iscan and colleagues.

The 20 remaining genes, except *CYP1B1*, *FMO5*, *NQO1* and *SUL2B1*, showed mRNA expression variation <10-fold between normal breast and liver. Another result was that *CYP1B1* and *CYP2D6* were significantly upregulated in ERα-negative (poorly differentiated) tumors relative to ERα-positive tumors. These two genes would thus correspond to markers of tumor aggressiveness.

Two genes (*GSTM1* and *GSTT1*) had undetectable mRNA expression in a number of breast tumors, and in normal breast tissues, probably owing to their particular polymorphism (a total absence of the two allele copies of these two loci in 'allele null' patients), although this needs to be confirmed at the DNA level. It is noteworthy that *GSTM1* and *GSTT1* polymorphisms are associated with the risk of breast cancer and that inherited metabolic variability may also influence breast cancer treatment outcome [31–33].

Finally, we identified seven genes of interest (*CYP2A6*, *CYP2B6*, *FMO5*, *NAT1*, *SULT2B1*, *GSTM3* and *ABCC11*) and further investigated their expression in a larger series of ERα-positive breast tumors. These genes either showed strong upregulation in ERα-breast tumors compared with normal breast tissue, suggesting a role in breast tumorigenesis, and/or showed upregulation in the ERα-positive tumors compared with the ERα-negative tumors, making them putative ERα-responsive genes.

It is noteworthy that due to lack of expression in breast tissue (*CYP2C9* and *CYP3A4*), due to no expression differences between normal and tumoral breast tissue (*FMO1*) and due to expression upregulation in ERα-negative compared with ERα-positive breast tumors (*CYP2D6*), these genes classically described to metabolize the tamoxifen in the liver were not further investigated in the 97 ERα-positive breast tumor series.

In the second part of this study we examined relationships between the expression status of these seven genes and the risk of disease recurrence and the response to tamoxifen therapy. The results point to *CYP2A6*, *CYP2B6*, *FMO5* and *NAT1* as new ERα-responsive genes, and point to *NAT1* as an independent predictor of response to tamoxifen. Indeed, expression levels of the *CYP2A6*, *CYP2B6*, *FMO5* and *NAT1* genes were strongly linked to *ERα* mRNA levels in our ERα-positive breast tumor series. Total validation of these four genes as effective ERα-responsive genes will require the use of classical *in vitro* or *in vivo* expression models, and the identification of estrogen-responsive elements within the promoters of the four genes.

The most important result of this study is that both univariate and multivariate prognostic analysis identified *NAT1* as both an independent prognostic factor of breast cancer relapse and as a putative predictor of the response to tamoxifen. The predictive value of *NAT1* in the response to endocrine therapy of breast cancer must now be confirmed in a prospective randomized study designed to show that this parameter influences outcome only in patients who receive adjuvant tamoxifen as compared with untreated patients. Indeed, previous epidemiological studies of the potential link between the *NAT1* genotype, breast cancer risk and lifestyle factors (including cooked meat and cigarettes) showed an increased risk among individuals with certain *NAT1* alleles who eat well-cooked meat [34]. We thus cannot rule out the possibility that the prognostic value of *NAT1* in our breast cancer series was due to individual variations in the metabolization of xenobiotics other than tamoxifen, influencing outcome independently of endocrine treatment.

Human aryl *N*-acetyltransferases are encoded by two genes (*NAT1* and *NAT2*) physically linked in chromosomal region 8p21.3-23.1. Despite their strong homology at the amino acid level (81%), *NAT1* and *NAT2* enzymes have distinct substrate specificity, although they do share certain substrates such as aromatic and heterocyclic amine carcinogens [35]. These enzymes also have distinct tissue expression profiles: *NAT2* is principally expressed in human liver and intestine, while *NAT1* is expressed more ubiquitously [36]. In normal breast tissue, NAT1 enzyme levels are high, while NAT2 enzyme levels are very low [37]. It is noteworthy that *NAT1* gene expression may reliably be studied at the mRNA level because *NAT1* mRNA expression detected by RT-PCR analysis seems to be highly associated with positive *NAT1* immunohistochemistry staining [37]. Immunochemistry-based studies show that NAT1 expression is strictly limited to epithelial cells, stromal tissues showing no NAT1 staining [37]. Few data are available on NAT1 expression in breast tumors. A recent study showed increased NAT1 enzyme activity in a series of 12 breast tumors as compared with normal breast tissue [38].

NAT1 overexpression was associated with good outcome in our cohort of ERα-positive postmenopausal breast cancer patients treated with adjuvant tamoxifen alone. We hypothesize that strong intratumoral *NAT1* expression could lead to increased detoxification of genotoxic and/or estrogenic tamoxifen metabolites, while having no action on the major antiestrogenic tamoxifen metabolites such as 4-hydroxy-tamoxifen, which is again metabolized by phase I enzymes (i.e. cytochromes P450).

Conclusions

In conclusion, this study points to a role of altered intratumoral expression of xenobiotic-metabolizing enzyme genes in breast tumorigenesis, identifies four putative ERα-responsive genes (*CYP2A6*, *CYP2B6*, *FMO5* and *NAT1*) and points to *NAT1* as an attractive candidate molecular marker predictive of antiestrogen responsiveness in breast cancer. This latter hypothesis is currently being tested in a large, prospective and homogeneous patient cohort.

Competing interests

None declared.

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Correspondence

Ivan Bièche, Laboratoire d'Oncogénétique – INSERM E0017, 35 rue Dailly, Centre René Huguenin, F-92211 St-Cloud, France. Tel: +33 1 47 11 15 66; fax: +33 1 47 11 16 96; e-mail: i.bieche@stcloud-huguenin.org