

Research article

Relationship between intratumoral expression of genes coding for xenobiotic-metabolizing enzymes and benefit from adjuvant tamoxifen in estrogen receptor alpha-positive postmenopausal breast carcinoma

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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 (ER α)-negative and ER α -positive breast tumors. Relevant genes were further investigated in a well-defined cohort of 97 ER α -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 ER α -positive breast tumors than in normal breast tissue, or showed higher mRNA levels in ER α -positive breast tumors than in ER α -negative breast tumors.

In the 97 ER α -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 ER α -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 (ER α). The presence of ER α 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 ER α -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; ER α = estrogen receptor alpha; *ESR1/ER α* = 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 ER α -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 ER α -negative and ER α -positive breast tumors. Seven relevant genes thus identified were further investigated in a well-defined cohort of 97 ER α -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; ER α -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*/ER α 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 ER α -negative tumors were also analyzed in order to investigate the relationship between target mRNA levels and ER α 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

| | Number of patients | Relapse-free survival | |
|-------------------------------------|--------------------|-----------------------|-----------------------|
| | | Relapses (%) | <i>P</i> ^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 '*Ntarget*'), were determined by the formula: $Ntarget = 2^{\Delta Ct_{sample}}$, 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 *Ntarget* values of the samples were subsequently normalized such that the mean of the *Ntarget* 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/ER α* gene (coding for the ER α) 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 μ l containing 1 \times RT buffer (500 μ M each dNTP, 3 mM MgCl₂, 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 μ M random hexamers (Pharmacia, Uppsala, Sweden) and 1 μ g 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 | | | |
| <i>CYP1A1</i> | NM_000499 | 15q24.1 | Cytochrome P450, subfamily IA, polypeptide 1 |
| <i>CYP1A2</i> | NM_000761 | 15q24.2 | Cytochrome P450, subfamily IA, polypeptide 2 |
| <i>CYP1B1</i> | NM_000104 | 2p21 | Cytochrome P450, subfamily IB, polypeptide 1 |
| <i>CYP2A6</i> | NM_000762 | 19q13.2 | Cytochrome P450, subfamily IIA, polypeptide 6 |
| <i>CYP2B6</i> | NM_000767 | 19q13.2 | Cytochrome P450, subfamily IIB, polypeptide 6 |
| <i>CYP2C9</i> | NM_000771 | 10q24.1 | Cytochrome P450, subfamily IIC, polypeptide 9 |
| <i>CYP2D6</i> | NM_000106 | 22q13.1 | Cytochrome P450, subfamily IID, polypeptide 6 |
| <i>CYP2E1</i> | NM_000773 | 10q24.3-qter | Cytochrome P450, subfamily IIE, polypeptide 1 |
| <i>CYP3A4</i> | NM_017460 | 7q22.1 | Cytochrome P450, subfamily IIIA, polypeptide 4 |
| <i>FMO1</i> | NM_002021 | 1q23-q25 | Flavin-containing monooxygenase 1 |
| <i>FMO5</i> | NM_018578 | 1q21 | Flavin-containing monooxygenase 5 |
| <i>LPO</i> | XM_042207 | 17q23.1 | Lactoperoxidase |
| Phase II enzymes | | | |
| <i>NQO1</i> | NM_000903 | 16q22.1 | NAD(P)H deshydrogenase, quinone 1 |
| <i>NAT1</i> | NM_000662 | 8p23.1-p21.3 | N-acetyltransferase 1 |
| <i>COMT</i> | NM_000754 | 22q11.21 | Catechol-O-methyltransferase |
| <i>EPHX1</i> | NM_000120 | 1q42.1 | Epoxyde hydrolase 1, microsomal |
| <i>SULT1A1</i> | NM_001055 | 16p12.1 | Sulfotransferase family, cytosolic, 1A, member 1 |
| <i>SULT2A1</i> | NM_003167 | 19q13.3 | Sulfotransferase family, cytosolic, 2A, member 1 |
| <i>SULT2B1</i> | NM_004605 | 19q13.3 | Sulfotransferase family, cytosolic, 2B, member 1 |
| <i>UGT1A1</i> | NM_000463 | 2q37 | UDP-glucuronosyltransferase, 1 family, polypeptide A1 |
| <i>GSTP1</i> | NM_000852 | 11q13 | Glutathion S-transferase pi 1 |
| <i>GSTM1</i> | NM_000561 | 1p13.3 | Glutathion S-transferase mu 1 |
| <i>GSTM3</i> | NM_000849 | 1p13.3 | Glutathion S-transferase mu 3 |
| <i>GSTT1</i> | NM_000853 | 22q11.23 | Glutathion S-transferase theta 1 |
| Phase III proteins | | | |
| <i>ABCB1</i> | NM_000927 | 7q21.1 | ATP-binding cassette, subfamily B (MDR/TAP), member 1 (MDR1) |
| <i>ABCC1</i> | NM_004996 | 16p13.1 | ATP-binding cassette, subfamily C (CFTR/MRP), member 1 (MRP1) |
| <i>ABCC11</i> | 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 ER α -negative and ER α -positive breast tumors

We analyzed the mRNA expression of 27 xenobiotic-metabolizing-enzyme genes, and the *MKI67* and *ESR1/ER α* genes, in a pool of normal liver tissue, in eight normal breast tissues, in five ER α -negative breast tumors and in 17 ER α -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 (\pm standard deviation) and ranges of mRNA levels for the 20 xenobiotic-metabolizing enzyme genes expressed in breast tissues, as well as for *ESR1/ER α* and for *MKI67*, are presented in Table 3. Target gene mRNA levels in the five ER α -negative breast tumors and in the 17 ER α -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 ER α -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 ER α -positive tumors compared with ER α -negative tumors.

It is noteworthy (Table 3) that *CYP1B1* and *CYP2D6* were significantly upregulated in ER α -negative tumors compared with ER α -positive tumors, identifying them as candidate markers of tumor aggressiveness in ER α -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 *Ntarget* 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 ER α -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 ER α -positive breast tumors from postmenopausal patients treated by surgery who only received tamoxifen hormone therapy 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 *Ntarget* values (calculated as described in Materials and methods) obtained for the eight normal breast RNA samples. The mean values for the eight normal breast *Ntarget* plus five standard deviations were considered to represent the

Table 3**mRNA levels of *MKI67*, *ESR1/ER α* and the 20 target genes expressed in breast tissues**

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

ER α , 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 *Ntarget* minus two standard deviations (or *Ntarget* 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 ER α -positive breast tumors

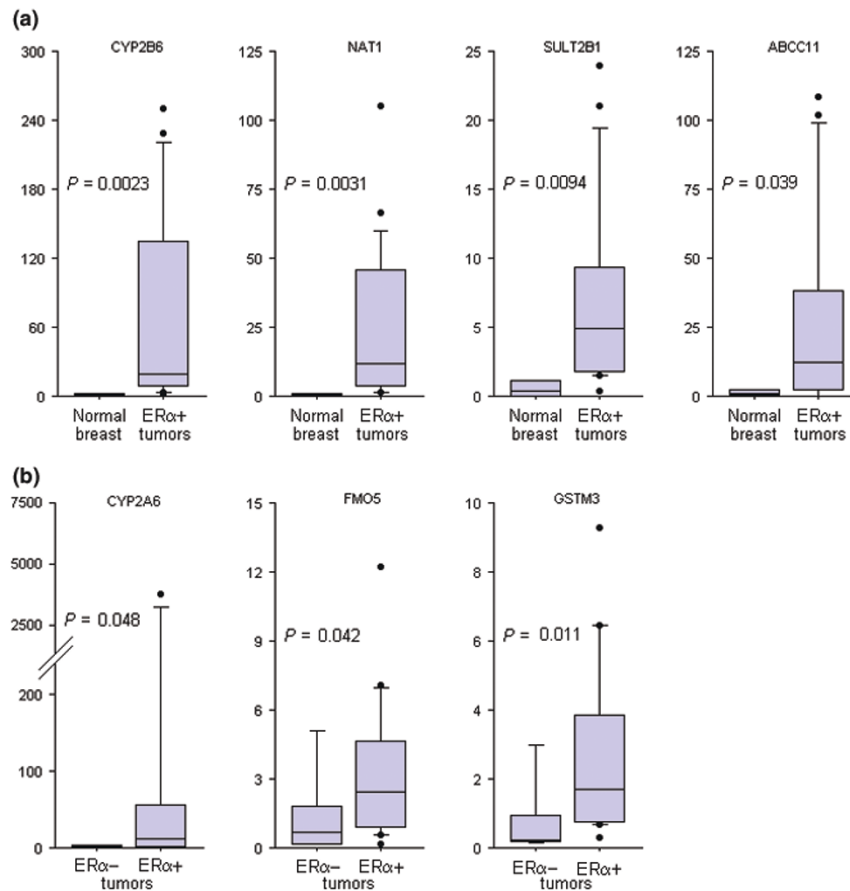
Using the Spearman rank correlation test (which compares continuous variables), we found a strong

positive correlation between *CYP2A6*, *CYP2B6*, *FMO5* and *NAT1* mRNA levels (Table 5). We also quantified *ESR1/ER α* mRNA levels in this series of 97 ER α -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 ER α -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 (ER α)-positive breast tumors. **(b)** Comparison between ER α -negative versus ER α -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**

| Gene | mRNA levels | | | Expression status | | |
|----------------|---------------------------------|--------|--------------|-----------------------------|------------------------|----------------------------|
| | Mean \pm SD | Median | Range | Underexpressed ^a | Normal | Overexpressed ^b |
| <i>CYP2A6</i> | 344.0 ^c \pm 1540.6 | 0.9 | 0.001–9741.1 | 15 (15.5) ^d | 58 (59.8) ^d | 24 (24.7) ^d |
| <i>CYP2B6</i> | 103.3 \pm 172.7 | 37.0 | 0.03–1053.1 | 1 (1.0) | 22 (22.7) | 74 (76.3) |
| <i>FMO5</i> | 5.2 \pm 6.3 | 2.6 | 0.10–30.1 | 1 (1.0) | 43 (44.3) | 53 (54.7) |
| <i>GSTM3</i> | 3.1 \pm 3.5 | 2.0 | 0.07–21.2 | 2 (2.1) | 60 (61.8) | 35 (36.1) |
| <i>SULT2B1</i> | 7.5 \pm 10.5 | 4.5 | 0.17–84.4 | 0 | 61 (62.9) | 36 (37.1) |
| <i>NAT1</i> | 46.5 \pm 55.4 | 21.5 | 1.1–295.6 | 0 | 20 (20.6) | 77 (79.4) |
| <i>ABCC11</i> | 37.1 \pm 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 *Ntarget* minus two standard deviations (SDs) (or *Ntarget* value = 0.1 when the latter calculation gave a negative value). ^b Greater than mean values for the normal breast *Ntarget* 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 *ER α* gene in the 97 estrogen receptor alpha (*ER α*)-positive breast tumor series**

| | <i>CYP2B6</i> | <i>FMO5</i> | <i>NAT1</i> | <i>SULT2B1</i> | <i>GSTM3</i> | <i>ABCC11</i> | <i>ESR1/ERα</i> |
|----------------|----------------------|-------------------------|----------------------|------------------------|------------------------|-------------------------|-----------------------------------|
| <i>CYP2A6</i> | + 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] |
| <i>CYP2B6</i> | | + 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] |
| <i>FMO5</i> | | | + 0.420 [< 0.001] | - 0.099 [NS (0.34)] | - 0.008 [NS (0.93)] | + 0.216 [0.032] | + 0.316 [0.0018] |
| <i>NAT1</i> | | | | + 0.077 [NS (0.46)] | + 0.104 [NS (0.31)] | + 0.041 [NS (0.69)] | + 0.293 [0.0037] |
| <i>SULT2B1</i> | | | | | + 0.232 [0.021] | + 0.049 [NS (0.64)] | + 0.091 [NS (0.38)] |
| <i>GSTM3</i> | | | | | | + 0.198 [0.049] | + 0.239 [0.018] |
| <i>ABCC11</i> | | | | | | | + 0.023 [NS (0.82)] |

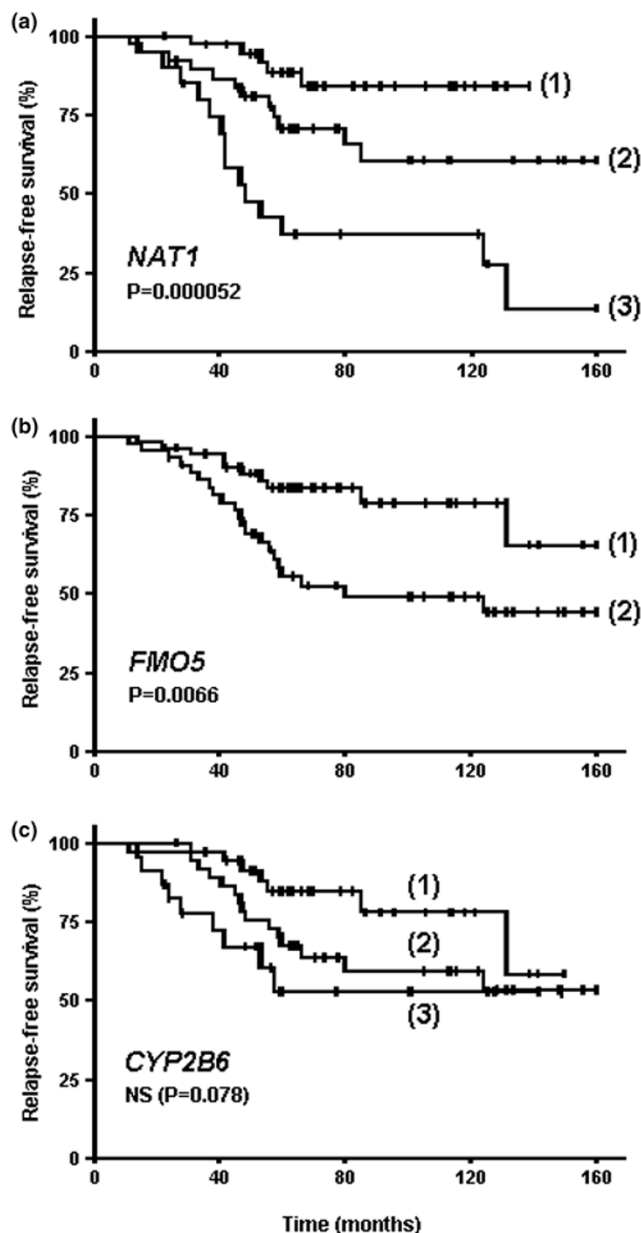
Data presented as Spearman rank correlation coefficient [*P* value (Spearman rank correlation test)]. NS, not significant.

Table 6**Relationships between the prognostic (\pm relapses) and the mRNA levels of the seven selected genes in 97 estrogen receptor alpha-positive breast tumors**

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

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

Table 7

Multivariate analysis of relapse-free survival

| Variable | Relapse-free survival | | |
|-------------------------|------------------------|------------------|--------|
| | Regression coefficient | RR (95% CI) | P^a |
| <i>NAT1</i> 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.

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 ER α -negative ($n=5$) and ER α -positive ($n=17$) breast tumors. Seven genes of interest were then further investigated in a well-defined cohort of 97 ER α -

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 *SULT2B1*, showed mRNA expression variation <10-fold between normal breast and liver. Another result was that *CYP1B1* and *CYP2D6* were significantly upregulated in ER α -negative (poorly differentiated) tumors relative to ER α -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 ER α -positive breast tumors. These genes either showed strong upregulation in ER α -breast tumors compared with normal breast tissue, suggesting a role in breast tumorigenesis, and/or showed upregulation in the ER α -positive tumors compared with the ER α -negative tumors, making them putative ER α -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 ER α -negative compared with ER α -positive breast tumors (*CYP2D6*), these genes classically described to metabolize the tamoxifen in the liver were not further investigated in the 97 ER α -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 ER α -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 ER α mRNA levels in our ER α -positive breast tumor series. Total validation of these four genes as effective ER α -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 metabolism 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]. Immunohistochemistry-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 ER α -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 ER α -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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