Oligonucleotide microarray analysis of estrogen receptor α -positive postmenopausal breast carcinomas: identification of *HRPAP20* and *TIMELESS* as outstanding candidate markers to predict the response to tamoxifen

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Abstract

The estrogen receptor α (ER α) status of breast tumors is used to identify patients who may respond to endocrine agents such as tamoxifen. However, ER α status alone is not perfectly predictive, and there is a pressing need for more reliable markers of endocrine responsiveness. In this aim, we used a two-step strategy. We first screened genes of interest by a pangenomic 44 K oligonucleotide microarray in a series of ten ER α -positive tumors from five tamoxifen-treated postmenopausal patients who relapsed (distant metastasis) and five tamoxifen-treated postmenopausal patients who did not relapse, matched with respect to age, Scarff–Bloom–Richardson grade, lymph node status, and macroscopic tumor size. Genes of interest ($n=24$) were then investigated in an independent well-characterized series of ER α -positive unilateral invasive primary breast tumors from postmenopausal women who received tamoxifen alone as adjuvant hormone therapy after primary surgery. We identified four genes (*HRPAP20*, *TIMELESS*, *PTPLB*, and *MGC29814*) for which high mRNA levels were significantly associated with shorter relapse-free survival (log-rank test). We also showed that hormone-regulated proliferation-associated 20 kDa protein (*HRPAP20*) and *TIMELESS* are 17 β -estradiol-regulated *in vitro* and are ectopically expressed in OH-Tam-resistant cell lines. In conclusion, these findings point to *HRPAP20* and *TIMELESS* as promising markers of tamoxifen resistance in women with ER α -positive breast tumors.

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Introduction

Tamoxifen is by far the most common endocrine agent used to treat women with all stages of breast cancer, and particularly for postmenopausal patients. Resistance to tamoxifen is a major problem. Estrogen receptor α (ER α) expression is used to identify breast cancer patients who are likely to respond to tamoxifen, but half of all patients with ER α -positive tumors fail to respond favorably to antiestrogen treatment (McGuire 1980). Moreover, the proportion of ER α -positive tumors is higher in postmenopausal patients than in younger patients, meaning that ER α status is poorly predictive in this population (Diab *et al.* 2000). Combined assessment of ER α and ER α -inducible genes (as markers of

functional ER α -mediated cell growth mechanisms) might be more reliable. Measurement of tumor progesterone receptor and PS2 expression is slightly more reliable than ER α assay alone in this setting. However, the expression of PR and PS2 correlates strongly with ER α expression, meaning that the PS2 and PR markers provide little further information on hormone dependence relative to ER α . There is thus a pressing need for more reliable markers of endocrine responsiveness.

The advent of efficient tools for large-scale gene expression analysis has provided new insights into the involvement of gene networks and regulatory pathways in various tumoral processes (DeRisi *et al.* 1996). cDNA and oligonucleotide microarrays are used to test the

expression pattern of thousands of genes at a time in individual tumors, and to identify genes or molecular signatures that are predictive of outcome (van't Veer *et al.* 2002).

Here, to identify candidate markers with which to predict the response to tamoxifen, we first used pangenomic dual color 44 K long oligonucleotide microarrays to screen ERα-positive tumors from ten postmenopausal breast cancer patients treated with adjuvant tamoxifen alone, of whom five relapsed (distant metastasis). Twenty-four genes of interest thus identified were then investigated in an independent panel of ERα-positive tumors from 48 tamoxifen-treated postmenopausal breast cancer patients, of whom 24 relapsed. Overexpression of four genes (*HRPAP20*, *TIMELESS*, *PTPLB*, and *MGC29814*) was associated with significantly shorter relapse-free survival in univariate analysis. We also found that *HRPAP20* and *TIMELESS* expression patterns differed between a hydroxy (OH)-Tam-sensitive cell line (MVLN) and two MVLN-derived OH-Tam-resistant cell lines.

Materials and methods

Patients and samples

We analyzed samples of 58 primary breast tumors excised from women at our institution from 1980 to 1994. Samples containing more than 70% of tumor cells were considered suitable for this study. Immediately following surgery, the tumor samples were placed in liquid nitrogen until RNA extraction. Ten tumors were used for oligonucleotide microarray analysis (screening set) and another 48 tumors were used for real-time quantitative RT-PCR (validation set).

The patients met the following criteria: primary unilateral non-metastatic postmenopausal breast carcinoma; ERα positivity (as determined at the protein level by biochemical methods (dextran-coated charcoal method until 1988 and enzymatic immunoassay thereafter) and confirmed by ERα real-time quantitative RT-PCR assay); complete clinical, histological, and biological information available; no radiotherapy or chemotherapy before surgery; and full follow-up at our institution. Standard prognostic factors are shown in Tables 1 and 2. The patients had physical examinations and routine chest radiography every 3 months for 2 years, then annually. Mammograms were done annually. The median follow-up was 7.2 years (range 1.5–10.0 years). All the patients received postoperative adjuvant endocrine therapy (20 mg tamoxifen daily for 3–5 years), and no other treatment. Twenty-nine patients relapsed. The distribution of first relapse events was as follows: 26 metastases and 3 local and/or regional recurrences with metastases.

We analyzed four ERα-positive cell lines (MCF-7, T-47D, ZR-75-1, and MDA-MB361) and five ERα-negative cell lines (SK-BR-3, HBL-100, MDA-MB157, MDA-MB231, and MDA-MB468), obtained from the American Tissue Type Culture Collection.

Specimens of adjacent normal breast tissue from four breast cancer patients and normal breast tissue from three women undergoing cosmetic breast surgery were used as sources of normal RNA.

For the analysis of genes of interest in normal adult human tissues, total RNAs from a variety of normal adult human tissues were purchased from Clontech. The tissues analyzed were breast, placenta, liver, spleen, testis, ovary, kidney, lung, trachea, heart, bone marrow, skeletal muscle, small intestine, thyroid, colon, thymus, prostate, uterus, salivary gland, leukocytes, bladder, spinal cord, adrenal gland, and brain.

Table 1 Characteristics of the ten estrogen receptor α (ERα)-positive tumors used in oligonucleotide microarray analysis (matched pairwise with respect to age, Scarff–Bloom–Richardson (SBR) grade, lymph node status, and macroscopic tumor size), from patients with and without relapse

	Age	Grade ^a	Nodes	Size ^b	Relapse	ER level ^c (fmol/mg)	PR level ^c (fmol/mg)
T1	68	2	8	30	—	351	623
T2	73	2	10	27	+	29	8
T3	71	2	1	22	—	56	52
T4	70	2	1	20	+	89	121
T5	60	2	1	32	—	284	77
T6	71	2	1	37	+	167	9
T7	65	2	4	30	—	505	93
T8	64	2	6	25	+	33	73
T9	63	3	2	26	—	19	187
T10	62	3	3	22	+	362	7

^aScarff–Bloom–Richardson classification.

^bMacroscopic tumor size (mm).

^cBiochemical values of the protein expression. Cutoff for ER and PR positivity was 15 fmol/mg protein. In consequence, tumors T2, T6, and T10 are considered as PR-negative tumors.

Table 2 Characteristics of the 48 estrogen receptor α (ER α)-positive tumors from patients with and without relapse, used for real-time RT-PCR analysis

	Number of patients	RFS	
		Number of events (%)	<i>P</i> value ^a
Age			
≤70	26	13 (50)	NS
>70	22	11 (50)	(0.46)
SBR histological grade ^b			
I+II	37	15 (40.5)	NS
III	11	9 (81.8)	(0.08)
Lymph node status			
≤3	31	13 (41.9)	NS
>3	17	11 (64.7)	(0.09)
Macroscopic tumor size			
≤30 mm	31	14 (45.1)	NS
>30 mm	17	10 (58.8)	(0.28)
PR status ^c			
Negative	19	12 (63.2)	NS
Positive	29	12 (41.4)	(0.13)
ER level			
Low (19–200 fmol/mg)	24	12 (50.0)	NS
High (200–783 fmol/mg)	24	12 (50.0)	(0.51)

^aLog-rank test. NS, not significant.^bScarff–Bloom–Richardson classification.^cCutoff for PR positivity was 15 fmol/mg protein.

Oligonucleotide microarray

We analyzed ten ER α -positive breast tumors, consisting of five tumors with relapses (distant metastasis) matched to five tumors without relapse with respect to age, Scarff–Bloom–Richardson (SBR) grade, lymph node status, and macroscopic tumor size (Table 1).

The quality of the ten ER α -positive breast tumor total RNAs, based on the 28S/18S rRNA ratio, was assessed by the RNA 6000 Nano Lab-On-chip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All specimens had a 28S-to-18S ratio higher than 1.5. A pool composed of equal amounts of total RNA from the ten ER α -positive breast tumors was used as the RNA reference. Five hundred nanograms of total RNA from each sample and from the RNA reference pool were used to generate labeled antisense cRNAs with T7 RNA polymerase.

We used the Agilent 44 K Whole Human Genome (G4112A) long (60 bp) oligonucleotide microarray and the dual-color analysis method in which probes from tumor samples and from reference RNA are differentially labeled with cyanine 5 and cyanine 3. These microarrays developed by Agilent Technologies have 44 290 features with 41 000 distinct oligonucleotides belonging to 33 715 sequences defined by their accession number. Due to the redundancy of probes and accession number, they represent around 18 795

symbols of Entrez_Gene (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>).

Oligonucleotide microarray experiments were performed with duplicates for each data point, using a dye-swap design to avoid dye bias (20 oligonucleotide microarray experiments in total): cRNA from each tumor was labeled with cyanine 3 (Cy3)-cytidine triphosphate (CTP) and the RNA reference pool with cyanine 5 (Cy5)-CTP for the direct comparison and vice versa for the dye-swap analysis.

Reverse transcription, linear amplification, cRNA labeling, and purification were performed with the Agilent linear amplification kit. The cRNA concentration and Cy3-CTP or Cy5-CTP incorporation were assessed with a u.v.-visible spectrophotometer. Hybridization was run for 17 h at 60 °C, with 1 µg Cy3- or Cy5-labeled cRNA from each tumor, mixed with the same amount of Cy5- or Cy3-labeled cRNA from the reference pool. The arrays were then washed with 0.6× and 0.01× SSC buffers containing Triton, and dried with a nitrogen gun. Microarrays were scanned with the Agilent DNA Microarray Scanner.

Feature Extraction software (Agilent Technologies) was used to quantify the intensity of fluorescent images and to normalize results by the local background subtraction option. Files used for statistical analysis contained, for each tumor sample, the list of 44 000 features associated with a set of values including the log ratio compared with the reference, the *P* value of the log ratio, and intensities. Dye-swap designs, in which each tumor is processed in duplicate, were used to assess reproducibility, and improved both the efficiency and the validity of the data. The final ratio (i.e., the fold change) is the average of the two individual ratios from the dye-swap experiment (combined experiments).

All data obtained by microarray analysis have been submitted to Array Express at the European Bioinformatics Institute (<http://www.ebi.ac.uk/arrayexpress/>) with accession number E-TABM-72. ArrayExpress (at the European Bioinformatics Institute) is a public repository for microarray data, which is aimed at storing well-annotated data in accordance with Microarray Gene Expression Data recommendations (<http://www.mged.org>).

Microarray data were mainly analyzed with Resolver software (Rosetta Inpharmatics, Seattle, WA, USA). All data were filtered to eliminate low-intensity values under 200 arbitrary units for both colors, a threshold determined on the basis of the linearity test. Each selected gene had at least a twofold change, with a *P* value <0.001. Using this procedure, 5234 genes passed the filter. For unsupervised clustering, we used a hierarchical agglomerative algorithm that pairs samples according to a Pearson-based distance.

ANOVA was applied to the microarray data in order to discriminate between breast tumors with and without relapse.

Real-time RT-PCR

Theoretical basis

Reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The larger the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The parameter threshold cycle (C_t) is defined as the fractional cycle number at which the fluorescence generated by SYBR green dye-amplicon complex formation passes a fixed threshold above baseline. The increase in the fluorescence signal associated with exponential growth of PCR products is detected by the laser detector of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), using PE Biosystems analysis software according to manufacturer's manuals.

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 two endogenous RNA control genes involved in two cellular metabolic pathways, namely TATA-binding protein (*TBP*; Genbank accession NM_003194), which encodes the TATA box-binding protein (a component of the DNA-binding protein complex transcription factor IID (TFIID), and *RPLP0* (also known as 36B4; NM_001002), which encodes human acidic ribosomal phosphoprotein P0. Each sample was normalized on the basis of its *TBP* (or *RPLP0*) content.

Results, expressed as N-fold differences in target gene expression relative to the *TBP* (or *RPLP0*) gene, and termed '*N_{target}*', were determined as $N_{target} = 2^{\Delta C_t \text{ sample}}$, where the ΔC_t value of the sample is determined by subtracting the average C_t value of the target gene from the average C_t value of the *TBP* (or *RPLP0*) gene (Bieche *et al.* 1999, 2001).

Primers and controls

Primers for *TBP*, *RPLP0*, and the target genes were chosen with the assistance of the Oligo 5.0 computer program (National Biosciences, Plymouth, MN, USA).

We conducted searches in the dbEST and nr databases to confirm the total gene specificity of the nucleotide sequences chosen as primers, and the absence of single nucleotide polymorphisms. In particular, the primer pairs were selected to be unique relative to the sequences

of closely related family member genes or of the corresponding retropseudogenes. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons. In general, amplicons were between 70 and 120 nucleotides long. Gel electrophoresis was used to verify the specificity of PCR amplicons.

For each primer pair, we performed no-template control (NTC) and no-reverse transcriptase control (RT-negative) assays, which produced negligible signals (usually >40 in C_t values), suggesting that primer-dimer formation and genomic DNA contamination effects were negligible.

RNA extraction, cDNA synthesis, and PCR conditions have been described elsewhere (Bieche *et al.* 2001).

Statistical analysis

As the mRNA levels did not fit a Gaussian distribution, a) the mRNA levels in each subgroup of samples were characterized by their median values and ranges, rather than their mean values and coefficients of variation and b) relationships between the molecular markers and clinical and biological parameters were tested by the non-parametric Mann-Whitney *U* test (Mann & Whitney 1947). Differences between two populations were judged significant at confidence levels $>95\%$ ($P < 0.05$).

To visualize the capacity of a given molecular marker to discriminate between two populations (in the absence of an arbitrary cutoff value), we summarized the data in a receiver operating characteristics (ROCs) curve (Hanley & McNeil 1982). These curves plot sensitivity (true-positives) on the y-axis against one-specificity (false-positives) on the x-axis, considering each value as a possible cutoff. The area under curve (AUC) was calculated as a single measure of the discriminatory capacity of each molecular marker. When a molecular marker has no discriminative value, the ROC curve lies close to the diagonal and the AUC is close to 0.5. When a marker has strong discriminative value, the ROC curve moves to the upper left-hand corner (or to the lower right-hand corner) and the AUC is close to 1.0 (or 0).

Relapse-free survival (RFS) was determined as the interval between diagnosis and detection of the first relapse (local and/or regional recurrence, and/or distant metastasis). Survival distributions were estimated by the Kaplan-Meier method (Kaplan & Meier 1958), and the significance of differences between survival rates was ascertained by the log-rank test (Peto *et al.* 1977).

Cell culture

We studied the following three human breast carcinoma cell lines in various pharmacological conditions:

an OH-Tam-sensitive cell line (MVLN) and two MVLN-derived OH-Tam-resistant cell lines (CL6·8 and CL6·32). MVLN is an ER α -positive and hormone responsive human breast carcinoma cell line derived from MCF-7 (Demirpence *et al.* 1993). Treatment of MVLN cells for 6 months led to the emergence of the OH-Tam-resistant (but still estrogen-dependent) individual cellular clones CL6·8 and CL6·32 (Badia *et al.* 2000) which have been characterized in a previous work (Vendrell *et al.* 2005).

MVLN, CL6·8, and CL6·32 cells were grown for one or two passages as previously described (Demirpence *et al.* 1993), then purged for 4 days in Dulbecco's modified Eagle medium without phenol red, supplemented with 3% of steroid-depleted, dextran-coated charcoal-treated fetal calf serum (DCC medium). Cells were then treated for 4 days (with one medium change) under the following pharmacological conditions: steroid-depleted medium (vehicle), 1 nM E₂ (17 β -estradiol), 200 nM OH-Tam, or both 1 nM E₂ and 200 nM OH-Tam.

Results

Screening of differentially expressed genes

Microarray experiments based on dual-color technology were performed with ten ER α -positive breast tumor RNAs (five tumors with relapse and five tumors without relapse), relative to an RNA reference pool prepared by mixing identical amounts of RNA from the same ten breast tumors. Each breast tumor sample was

analyzed in duplicate using a dye-swap design (20 oligonucleotide microarray experiments in total).

Using Resolver software, an intensity- and fold change-based filtering approach (greater than twofold change and P value <0.001) selected 5234 features from the 44 290 present on the 60-mer oligonucleotide microarray. The unsupervised hierarchical clustering of these 5234 features is presented in Fig. 1. The duplicates (dye-swap) of each breast tumor sample clustered tightly together. On this unsupervised hierarchy, the samples fell into two subgroups: one contained four breast tumors, of which one (25%) was associated with relapse, while the second contained the other six breast tumors, four (67%) of which were associated with relapse.

Using ANOVA, 105 genes were found to be differentially expressed ($P<0.001$) between breast tumors with and without relapse (supplementary data see <http://jme.endocrinology-journals.org/content/vol39/issue4>). Among this set of 105 genes, only 24 genes perfectly discriminated between the ER α -positive breast tumors with and without relapse (AUC-ROC, 1.000). The expression of 13 genes was increased in ER α -positive breast tumors with relapse, while expression of 11 genes was decreased (Table 3).

mRNA expression of *ESR1/ER α* , *MKI67*, and the 24 candidate genes in 24 patients who relapsed and 24 patients who did not relapse

The expression level of the 24 genes identified by oligonucleotide microarray screening and discriminating perfectly between breast tumors with and without relapse was then determined by real-time

Figure 1 Unsupervised hierarchical clustering of ten ER α -positive breast tumors (T1–T10) tested in duplicate with a dye-swap design (20 oligonucleotide microarray experiments in total). The classification was determined by the 5234 filtered features, with an agglomerative algorithm based on the Pearson correlation coefficient. R+, relapse; R–, no relapse; and -dw, dye-swap replicate.

Table 3 Genes perfectly discriminated between five estrogen receptor α (ER α)-positive breast tumors with relapse and five breast tumors without relapse

	Genbank accession number	Chromosomal location	Gene definition
Genes			
Upregulated genes (<i>n</i> = 13)			
<i>ANAPC1</i>	NM_022662	2q12·1	Anaphase promoting complex subunit 1
<i>AY062331</i>	AY062331	2p11·2	Homo sapiens clone 105/707 immunoglobulin light-chain κ variable region
<i>CPM</i>	NM_001874	12q14·3	Homo sapiens carboxypeptidase M
<i>CTSL2</i>	NM_001333	9q22·2	Homo sapiens cathepsin L2
<i>HRPAP20</i>	NM_014165	5q21	Hormone-regulated proliferation-associated protein 20
<i>MGC29814</i>	NM_182565	17q25·1	Homo sapiens hypothetical protein MGC29814
<i>NEK2</i>	NM_002497	1q32·2-q41	Homo sapiens NIMA (never in mitosis gene a)-related kinase 2
<i>PTPLB</i>	NM_198402	3q21·1	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b
<i>RANBP2L1</i>	NM_005054	2q13	Homo sapiens RAN-binding protein 2-like 1 (RANBP2L1), transcript variant 1
<i>TIMELESS</i>	NM_003920	12q12-q13	Homo sapiens timeless homolog (<i>Drosophila</i>)
<i>TNFAIP2</i>	NM_006291	14q32	Homo sapiens tumor necrosis factor, α -induced protein 2
<i>TTK</i>	NM_003318	6q13-q21	Homo sapiens TTK protein kinase
<i>UHRF1</i>	NM_013282	19p13·3	Homo sapiens ubiquitin-like, containing PHD and RING finger domains, 1
Downregulated genes (<i>n</i> = 11)			
<i>ACAA1</i>	NM_001607	3p23-p22	Homo sapiens acetyl-coenzyme A acyltransferase 1
<i>ALB</i>	NM_000477	4q11-q13	Homo sapiens albumin
<i>AUH</i>	NM_001698	9q22·31	Homo sapiens AU RNA-binding protein/enoyl-coenzyme A hydratase
<i>LOC441777</i>	XM_497521	16q24·1	Homo sapiens LOC441777
<i>LOC440765</i>	XM_498851	20q13·13	Homo sapiens LOC440765
<i>LOC283663</i>	XM_378514	15p21·3	Homo sapiens LOC283663
<i>LRP2</i>	NM_004525	2q24-q31	Homo sapiens low-density lipoprotein-related protein 2
<i>MPHOSPH6</i>	NM_005792	16q23·3	Homo sapiens M-phase phosphoprotein 6
<i>PPP2R2C</i>	NM_020416	4p16·1	Homo sapiens protein phosphatase 2, regulatory subunit B (PR 52), γ -isoform
<i>RGN</i>	NM_004683	Xp11·3	Homo sapiens regucalcin (senescence marker protein 30)
<i>SYNPO2L</i>	NM_024875	10q22·2	Synaptopodin 2-like

RT-PCR in an independent cohort of 24 ER α -positive breast tumor patients who relapsed and 24 ER α -positive breast tumor patients who did not relapse (Table 2). All these 48 ER α -positive tumors were from postmenopausal patients treated with primary surgery followed by adjuvant tamoxifen alone. Six (46%) of the 13 upregulated genes identified by oligonucleotide microarray analysis were significantly upregulated in the 24 individual ER α -positive breast tumors with relapse, as compared with the 24 ER α -positive breast tumors without relapse ($P<0.05$; Table 4). The six genes significantly upregulated were *HRPAP20*, *TIMELESS*, *NEK2*, *PTPLB*, *MGC29814*, and *RANBP2L1* (Table 4). The capacity of each of these six genes to discriminate between breast tumors with and without relapse was then tested by ROC curve analysis. The overall diagnostic values of the six molecular markers were assessed in terms of their AUC values (Table 4). *HRPAP20* emerged as

the most discriminatory marker of relapse status (ROC-AUC, 0.911).

In the same set of 48 ER α -positive tumors, we also examined the expression of the *ESR1/ER α* gene and the *MKI67* gene (encoding the proliferation-related Ki-67 antigen) and found that expression of these two genes was similar in the ER α -positive breast tumors with and without relapse (AUC-ROC, 0.468 and 0.524 respectively), suggesting that the six genes were associated with outcome independently of proliferation and ER α expression status.

None of the 11 downregulated genes identified by oligonucleotide microarray analysis was significantly downregulated in the 24 breast tumors with relapse as compared with the 24 tumors without relapse.

The mRNA levels indicated in Table 4 (calculated as described in Materials and methods) show the abundance of the target relative to the endogenous control (*TBP*) used to normalize the starting amount

Table 4 Statistical analysis of mRNA expression of genes in 24 estrogen receptor α (ER α)-positive breast tumors with relapse relative to 24 ER α -positive breast tumors without relapse

	Genbank	Breast tumors without relapse (n=24)	Breast tumors with relapse (n=24)	P ^a	ROC-AUC ^b
Genes					
<i>HRPAP20</i>	NM_014165	1.0 (0.56–1.65) ^c	1.93 (0.52–3.58)	0.000 001	0.911
<i>TIMELESS</i>	NM_003920	1.0 (0.46–2.79)	1.88 (0.41–3.84)	0.0008	0.782
<i>NEK</i>	NM_002497	1.0 (0.21–5.05)	2.12 (0.77–8.67)	0.001	0.779
<i>PTPLB</i>	NM_198402	1.0 (0.54–2.21)	1.45 (0.73–3.62)	0.003	0.746
<i>MGC29814</i>	NM_182565	1.0 (0.40–3.18)	1.51 (0.53–5.98)	0.006	0.731
<i>RANBP2L1</i>	NM_005054	1.0 (0.07–3.48)	1.31 (0.33–2.46)	0.04	0.678
<i>ACAA1</i>	NM_001607	1.0 (0.37–1.57)	1.21 (0.51–4.14)	0.06	0.662
<i>AUH</i>	NM_001698	1.0 (0.35–2.65)	1.26 (0.50–2.72)	0.09	0.643
<i>CTSL2</i>	NM_001333	1.0 (0.28–6.96)	1.67 (0.22–9.28)	0.17	0.615
<i>CPM</i>	NM_001874	1.0 (0.11–5.15)	1.37 (0.38–13.92)	0.24	0.600
<i>ANAPC1</i>	NM_022662	1.0 (0.62–2.65)	1.67 (0.22–9.28)	0.29	0.589
<i>MPHOSPH6</i>	NM_005792	1.0 (0.30–5.26)	1.22 (0.40–4.01)	0.37	0.576
<i>LOC440765</i>	XM_498851	1.0 (0.02–163.2)	2.44 (0.10–145.6)	0.42	0.568
<i>LOC441777</i>	XM_497521	1.0 (0.01–110.7)	1.23 (0.01–1109)	0.43	0.566
<i>UHRF1</i>	NM_013282	1.0 (0.17–8.05)	1.08 (0.39–4.07)	0.69	0.534
<i>AY062331</i>	AY062331	1.0 (0.02–7.49)	0.64 (0.03–86.52)	0.82	0.519
<i>ALB</i>	NM_000477	1.0 (0.08–23.48)	0.75 (0.14–20.80)	0.90	0.510
<i>LOC283663</i>	XM_378514	1.0 (0.28–5.49)	0.90 (0.15–6.74)	0.97	0.497
<i>SYNPO2L</i>	NM_024875	1.0 (0.00–76.11)	0.67 (0.00–28.15)	0.74	0.472
<i>TNFAIP2</i>	NM_006291	1.0 (0.24–3.04)	0.90 (0.37–6.10)	0.62	0.458
<i>LRP2</i>	NM_004525	1.0 (0.00–19.00)	1.08 (0.01–13.07)	0.49	0.442
<i>RGN</i>	NM_004683	1.0 (0.20–32.40)	0.75 (0.01–16.37)	0.46	0.438
<i>TTK</i>	NM_003318	1.0 (0.08–4.01)	0.55 (0.10–2.18)	0.23	0.398
<i>PPP2R2C</i>	NM_020416	1.0 (0.01–4.08)	0.11 (0.00–5.03)	0.17	0.384
<i>ESR1/ERα</i>	NM_000125	1.0 (0.10–2.89)	0.90 (0.14–2.91)	0.68	0.468
<i>MKI67</i>	NM_002417	1.0 (0.11–4.50)	1.10 (0.30–5.71)	0.77	0.524

^aMann–Whitney's *U* test.

^bROCs (receiver operating characteristics); AUC (area under curve) analysis.

^cMedian (range) of gene mRNA levels.

and quality of total RNA. Similar results were obtained with a second endogenous control, *RPLP0* (also known as 36B4). Indeed, the six upregulated genes were also significantly upregulated in tumor samples from the patients who relapsed relative to the patients who did not relapse.

Prognostic value of *HRPAP20*, *NEK2*, *TIMELESS*, *PTPLB*, *MGC29814*, and *RANBP2L1*

We used univariate analysis (log-rank test) to further study the prognostic value of *HRPAP20*, *NEK2*, *TIMELESS*, *PTPLB*, *MGC29814*, and *RANBP2L1*. For each gene, the 48 ER α -positive breast tumors were divided into two groups of 24 tumors with 'low' and 'high' mRNA levels. Univariate analysis showed that a high expression level of *HRPAP20*, *TIMELESS*, *PTPLB*, and *MGC29814* correlated with significantly shorter RFS (Fig. 2A–D respectively). The outcomes of the 24 patients with high mRNA levels of these four genes were significantly worse than those of the 24 patients with low mRNA levels. No significant prognostic value

was associated with the two other genes, *NEK* and *RANBP2L1* (Fig. 2E and F respectively).

mRNA expression of *HRPAP20*, *TIMELESS*, *PTPLB*, and *MGC29814* in the OH-Tam-sensitive cell line MVLN and the OH-Tam-resistant cell lines CL6-8 and CL6-32

To further investigate the potential endocrine responsiveness function of *HRPAP20*, *TIMELESS*, *PTPLB*, and *MGC29814* in ER α -positive breast cancer, we analyzed mRNA levels of these four genes by real-time RT-PCR in MVLN, CL6-8, and CL6-32 cells after 4 days of E₂ and/or OH-Tam treatment.

mRNA expression of *PTPLB* and *MGC29814* did not vary in any of the cells lines during any of the treatments.

Figure 3 illustrates compiled expression data for the other two genes (*HRPAP20* and *TIMELESS*), as well as the well-known ER α -induced gene *pS2/TFF1*, in the three cell lines after pharmacological treatment. As *pS2/TFF1*, *HRPAP20*, and *TIMELESS* responded positively to E₂ in the three cell lines (greater than twofold upregulation).

Figure 2 RFS curves for tumors with low target gene expression ($n=24$) and high target gene expression ($n=24$).

In the OH-Tam-sensitive MVLN cells, the E_2 response was fully antagonized in the presence of OH-Tam, and OH-Tam displayed a 'reverse pharmacology', characterized by the negative regulation of both the

TIMELESS gene expression and to a less extent of the *HRAP20* gene expression.

Interestingly, the reverse pharmacology of OH-Tam on these two genes was lost in the two OH-Tam-

Figure 3 mRNA levels of *HRPAP20*, *TIMELESS*, and *pS2/TFF1* in MVLN, CL6-8, and CL6-32 cells after 4 days of culture in steroid-depleted medium (vehicle), or with E₂ and/or OH-Tam. For each gene and each cell line, mRNA levels were normalized such that the value of the vehicle sample was 1. The mRNA levels indicated are means from at least three independent experiments.

Figure 4 mRNA levels of *HRPAP20*, *TIMELESS*, and *pS2/TFF1* in MVLN after 8 h, 18 h, and 4 days of culture in steroid-depleted medium (vehicle) or with E₂. For each gene, mRNA levels of each E₂-treated sample were normalized such that the value of the vehicle sample was 1. The mRNA levels indicated are means from at least three independent experiments.

resistant cell lines CL6·8 and CL6·32. Indeed, in the CL6·8 or CL6·32 cells, a new OH-Tam response emerged, characterize either by no activity on *HRPAP20* or *TIMELESS* gene expression under OH-Tam treatment alone (*HRPAP20* gene expression in CL6·8 and CL6·32 cell and *TIMELESS* gene expression in CL6·32 cells; Fig. 3) or by the transformation of the reverse pharmacological action of OH-Tam in an agonist ('estrogen-like') activity (*TIMELESS* gene expression in the CL6·8 cells; Fig. 3). Finally, when the cell lines were treated with both E₂ and OH-Tam, OH-Tam counteracted the E₂-induced upregulation of *HRPAP20* and *TIMELESS* in MVLN cells but not in the two OH-Tam-resistant cell lines, CL6·8 and CL6·32.

To further know whether *HRPAP20* and *TIMELESS* genes are under direct or indirect regulation of the ER, we analyzed *HRPAP20*, *TIMELESS*, and *pS2/TFF1* gene expression in the MVLN cell line treated for 4 days, but also following two shorter E₂ treatments, i.e., 6 and 18 h. Figure 4 shows that *HRPAP20* and *TIMELESS* genes responded early (from 6 h) to E₂ in the MVLN cell line, these results suggest that, as *pS2/TFF1*, *HRPAP20*, and *TIMELESS* genes are regulated directly by estrogen.

Taken all together, these data suggest the involvement of ER-dependent signaling in the E₂ regulation of *HRPAP20* and *TIMELESS* genes.

mRNA expression of *HRPAP20* and *TIMELESS* in breast tumor cell lines

The expression level of *HRPAP20* and *TIMELESS* was then determined in nine well-characterized breast tumor cell lines, including four ER α -positive cell lines (MCF-7, T-47D, ZR-75-1, and MDA-MB361) and five ER α -negative cell lines (SK-BR-3, HBL-100, MDA-MB157, MDA-MB231, and MDA-MB468; Fig. 5).

HRPAP20 was upregulated (greater than threefold the median value in seven normal human breast tissue samples) in MDA-MB361 and MDA-MB468 cells. *TIMELESS* was upregulated in two ER α -positive cell lines (T-47D and MDA-MB361) and three ER α -negative cell lines (HBL-100, MDA-MB157, and MDA-MB468). None of the human breast cell lines showed *HRPAP20* or *TIMELESS* downregulation (less than threefold the median value in seven normal human breast tissue samples).

mRNA expression of *HRPAP20* and *TIMELESS* in normal human tissues

Knowledge of the physiological expression of genes of interest can help to detect abnormal expression in pathological situations. We therefore assessed *HRPAP20* and *TIMELESS* mRNA levels in a collection of 24 normal human tissues (Table 5). The expression of *HRPAP20* and *TIMELESS* was readily detected in all the tissues.

Figure 5 mRNA levels of *HRPAP20* and *TIMELESS* in nine breast tumor cell lines. For each gene, mRNA levels were normalized such that the median value of seven normal human breast tissue samples was 1. The mRNA levels indicated are means from at least three independent experiments.

The highest levels of *HRPAP20* expression (greater than threefold the value for normal breast tissue) were found in kidney and skeletal muscle, and the lowest level (less than threefold the value for normal breast tissue) was found in lung tissue. The highest expression levels for *TIMELESS* were found in liver, bone marrow, and thymus, and the lowest level was found in skeletal muscle.

Discussion

In an attempt to identify new candidate molecular markers with which to predict the antiestrogen responsiveness of ER α -positive postmenopausal breast cancer, we first used an oligonucleotide microarray approach to measure the expression of a large panel of probes ($n = 44\,290$ corresponding to a minimum of

Table 5 mRNA expression of *HRPAP20* and *TIMELESS* in normal human tissues

	<i>HRPAP20</i>	<i>TIMELESS</i>
Tissue		
Breast	1.00	1.00
Placenta	0.84 ^a	1.52
Liver	1.95	4.69
Spleen	1.54	1.75
Testis	1.26	1.39
Ovary	2.63	0.99
Kidney	3.76	1.94
Lung	0.18	1.25
Trachea	1.10	1.43
Heart	2.98	1.14
Bone marrow	0.68	4.86
Skeletal muscle	8.66	0.28
Small intestine	2.45	2.89
Thyroid	1.74	0.94
Colon	2.74	2.50
Thymus	0.89	5.10
Prostate	2.02	0.95
Uterus	1.03	1.05
Salivary gland	2.10	0.86
Leukocyte	2.56	2.36
Bladder	1.81	1.16
Spinal cord	2.22	1.26
Adrenal gland	2.67	1.43
Brain	2.10	0.61

^amRNA levels were normalized such that the breast tissue value was 1.

18 795 well-known genes) in ten ER α -positive breast tumors from patients who relapsed ($n=5$) or did not relapse ($n=5$). Given the relatively small number of the tumor samples, we duplicated the microarray experiments by a dye-swap design. This screening step identified 24 genes that each perfectly discriminated between breast tumors with and without relapse (AUC–ROC, 1.000). These 24 genes of interest were then further investigated in an independent well-defined cohort of 48 ER α -positive postmenopausal breast cancer patients treated with primary surgery followed by adjuvant tamoxifen alone; 24 of these patients had relapsed and 24 had not relapsed. Among the 24 genes identified by oligonucleotide microarray analysis, six (*HRPAP20*, *NEK2*, *TIMELESS*, *PTPLB*, *MGC29814*, and *RANBP2L1*) were significantly upregulated in the 24 patients who relapsed as compared with the 24 patients who did not relapse ($P<0.05$; Table 2). Univariate analysis showed that, among these six genes, only *HRPAP20*, *TIMELESS*, *PTPLB*, and *MGC29814* correlated with RFS (Fig. 2A–D).

This two-step strategy was by no means exhaustive, and many possibly relevant genes were certainly missed, but it nevertheless demonstrates that several potentially useful marker genes can be identified in a limited number of microarray experiments.

None of the four genes identified in this study is included in the recently characterized gene expression

signatures that predict the clinical outcome of breast cancer patients treated with tamoxifen, i.e., the 44-gene signature by Jansen *et al.* (2005), the 21-gene signature by Paik *et al.* (2004) and the two-gene signature by Ma *et al.* (2004). However, it is difficult to compare our results with those of these three studies, because of differences in the study populations, techniques and materials used. For example, contrary to the authors of these previous studies, we chose to analyze a well-defined cohort of postmenopausal patients with ER α -positive breast cancer treated with primary surgery followed by adjuvant tamoxifen alone.

It is noteworthy that we measured the expression of *HOXB13* and *IL17BR* characterized by Ma *et al.* (2004) in our panel of 48 tumors. In agreement with Reid *et al.* (2005), we found that *HOXB13* and *IL17BR* expression was similar in the 24 patients who relapsed and the 24 patients who did not relapse: AUC–ROC, 0.505 and 0.482 respectively (data not shown).

Our data identify *HRPAP20*, *TIMELESS*, *PTPLB*, and *MGC29814* as candidate prognostic markers in tamoxifen-treated postmenopausal breast cancer patients. However, it remains to be determined whether these four genes predict the tumor response to tamoxifen and/or reflect intrinsic tumor aggressiveness. A prospective randomized study is needed to show whether these molecular markers influence outcome only in patients who receive adjuvant tamoxifen or also in untreated patients. To further investigate the potential endocrine responsiveness function of *HRPAP20*, *TIMELESS*, *PTPLB*, and *MGC29814* in ER α -positive breast cancer, we analyzed their expression in an OH-Tam-sensitive cell line and in two OH-Tam-resistant cell lines (Badia *et al.* 2000, Vendrell *et al.* 2005). We found that two of the four genes (*HRPAP20* and *TIMELESS*) are under direct regulation of E₂ (Figs 3 and 4). Moreover, the E₂ response was abolished by OH-Tam treatment in the OH-Tam-sensitive cell line (antagonistic action of OH-Tam), but not in the two OH-Tam-resistant cell lines (agonistic action of OH-Tam). Taken together, these findings suggest that *HRPAP20* and *TIMELESS* might encode proteins with a critical role in the endocrine responsiveness pathway, but little or nothing is known of the relevance of these two genes in breast cancer biology.

We found only two studies of *HRPAP20* from the same team in PubMed journals (Karp *et al.* 2004, 2007). Interestingly, these authors identified *HRPAP20* as a novel phosphoprotein that enhances the growth and survival of hormone responsive tumor cells (Karp *et al.* 2004). *HRPAP20* was identified with a differential mRNA display approach, in a prolactin-dependent rat Nb2 T lymphoma cell line stimulated with various hormones and differentiating agents. Stable transfection of *HRPAP20* into MCF-7 cells significantly increased proliferation in the absence of hormone

stimulation and augmented survival in the absence of serum (Karp *et al.* 2004). Very recently, these authors also suggested that HRPAP20 is an important regulator of breast cancer cell invasion by a calmodulin-mediated mechanism leading to increased matrix metalloproteinase 9 secretion (Karp *et al.* 2007). We also found that HRPAP20 is ubiquitously expressed in human tissues and upregulated in two of the nine breast tumor cell lines tested (MDA-MB361 and MDA-MB468). Finally, *in silico* analysis of the Oncomine database (<http://www.oncomine.org/>), that catalogs 300 primary research articles on the cancer transcriptome, showed that HRPAP20 was upregulated in 18 breast cancer 1 (BRCA1) mutation-associated breast tumors as compared with 97 sporadic breast tumors ($P=0.0005$), based on microarray data from van't Veer *et al.* (2002).

Little is also known of the role of TIMELESS in cancer biology. Timeless protein is mainly known for its essential role in the circadian rhythm of *Drosophila*. However, recent human studies suggest an intimate connection between the circadian cycle and DNA damage checkpoints, that is partly mediated by timeless protein (Unsal-Kacmaz *et al.* 2005). The latter authors also showed that timeless protein interacts with Chk1 kinase, which regulates DNA damage-induced G2/M arrest and is mainly activated by BRCA1 (Yarden *et al.* 2002). Finally, a recent study (Naderi *et al.* 2007) identified TIMELESS among a common prognostic signature of 29 genes that associated with survival in breast cancer in three major independent datasets: their cohort (Naderi *et al.* 2007), the van de Vijver *et al.* (2002) dataset, and the Wang *et al.* (2005) dataset.

In the present study, we found that TIMELESS is ubiquitously expressed in human tissues and upregulated in half the breast tumor cell lines tested.

In conclusion, this study points to HRPAP20 and TIMELESS as attractive candidate molecular markers for predicting the tamoxifen responsiveness of ER α -positive postmenopausal breast cancer. Further studies are necessary to discover whether these two markers may also be useful in premenopausal breast cancer qualifying for tamoxifen therapy, or for predicting the response to other endocrine agents such as aromatase inhibitors, other selective ER modulators, and progestins. These possibilities are currently being tested in large prospective clinical studies.

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