Advances in Brief

Expression Analysis of Estrogen Receptor α Coregulators in Breast Carcinoma: Evidence That *NCOR1* Expression Is Predictive of the Response to Tamoxifen[1]

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Abstract

Purpose: **Dysregulated expression of steroid receptor transcriptional coactivators and corepressors has been implicated in tamoxifen resistance, especially in estrogen receptor (ER) α-positive breast cancer patients. Therefore, expression analysis of these ERα coregulators may identify new predictors of the response to tamoxifen treatment.**

***Experimental Design*: We measured mRNA levels of 16 coactivator and 11 corepressor genes with a real-time quantitative reverse transcription-PCR method in 14 ERα-positive breast tumors. Three selected coactivator genes (*TIF2*, *AIB1*, and *GCN5L2*) and two corepressor genes (*NCOR1* and *MTA1L1*) were additionally investigated in a well-characterized series of ERα-positive unilateral invasive primary breast tumors from 99 postmenopausal patients who only received tamoxifen as adjuvant hormone therapy after primary surgery. We sought relationships between mRNA levels of the coregulators and those of molecular markers, including *ERα*, *ERβ*, *CCND1*, and *ERBB2*.**

***Results*: ERα coregulator expression was unrelated to age, histological grade, lymph node status, and macroscopic tumor size. The relationship between mRNA expression of the coregulators, and *ERα* and β only showed a significant positive correlation between *GCN5L2* and *ERα* ($P = 0.015$). mRNA levels of *CCND1* correlated with those of all of the coregulators studied ($P < 0.05$ or trend), whereas *ERBB2* mRNA levels only correlated with *AIB1* mRNA levels ($P = 0.011$). Low *NCOR1* expression (*versus* intermediate and high) was associated with significantly shorter relapse-free survival (log-rank test; $P = 0.0076$). The prognostic significance of low *NCOR1* expression persisted in Cox multivariate regression analysis ($P = 0.043$).**

***Conclusions*: These findings point to *NCOR1* as a promising independent predictor of tamoxifen resistance in patients with ERα-positive breast tumors.**

Introduction

ER[3] α belongs to the superfamily of steroid nuclear receptor transcriptional factors. It regulates the proliferation and differentiation of many tissues, especially reproductive tissues (1). On binding to specific DNA sequences such as EREs, estrogen-ERα complexes activate or repress target gene transcription. The biological activity of estrogen is now realized to be more complex than initially thought, with the discovery of a second ER named ERβ (2). The ER-mediated transcriptional activity of estrogen is influenced by several regulatory factors known as coactivators and corepressors, which activate or repress the transcription of ER-responsive genes (1). Up to now, the enhancer activity of these coregulators has mainly been studied with ERα, but it may differ between the two ERs, providing additional regulatory steps in estrogen signaling (3). Transcriptional activation is triggered, via histone acetyltransferase activity, by protein-protein interactions between the receptor and cofactors, and also through interactions with the SWI/SNF chromatin remodeling complex; together, these mechanisms result in nucleosome disruption and allow RNA polymerase complex transcription activity to take place (4). Transcription silencing is also based on protein-protein interactions, this time between the receptor and corepressors, the latter belonging to larger protein complexes with histone deacetylase activity, which promotes nucleosome condensation (5).

Tamoxifen is the most common endocrine agent used at all stages of breast cancer and particularly for the treatment of postmenopausal patients. ERα status has been used to identify breast cancer patients who are likely to respond to tamoxifen, but resistance nonetheless occurs in 50% of treated ERα-positive breast cancer patients (6). This tamoxifen resistance could be because of dysregulation of ERα coregulator expression in breast tumors (7). Indeed, in addition to the competitive antag-

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[3] The abbreviations used are: ER, estrogen receptor; ERE, estrogen-responsive element; RT-PCR, reverse transcription-PCR; TIF2, transcriptional intermediary factor 2; AIB1, augmented in breast cancer 1; GCN5L2, general control of amino acid synthesis, yeast, homologue-like 2; NCOR1, nuclear receptor corepressor 1; MTA1L1, metastasis-associated 1-like 1; PR, progesterone receptor; Ct, cycle threshold; TBP, TATA box-binding protein; RFS, relapse-free survival.

Table 1 Coregulator genes mRNA levels in 14 ERα-positive breast tumors

Genes	Genbank accession no.	Mean ± SD	Range
Coactivators			
SRC1 (NCOA1)	NM_003743	1.7 ± 0.6	1–2.8
TIF2[a] *(NCOA2)*	NM_006540	6.0 ± 4.7	1–17.6
AIB1 *(NCOA3)*	NM_006534	1.8 ± 0.6	1–3.1
ARA70 (NCOA4)	NM_005437	2.3 ± 0.8	1–4.4
ARA54 (RNF14)	NM_004290	2.2 ± 1.0	1–4.6
TIF1	NM_003852	3.1 ± 1.8	1–6.9
CARM1	XM_032719	3.7 ± 2.3	1–8.9
SRCAP	NM_006662	2.4 ± 1.1	1–5.1
P300 (EP300)	NM_001429	2.3 ± 1.5	1–5.8
CBP (CREBBP)	NM_004380	3.5 ± 1.9	1–7.7
GCN5L2	NM_021078	4.0 ± 3.4	1–13.5
PCAF	NM_003884	4.2 ± 4.1	1–4.9
BRG1 (SMARCA4)	NM_003072	1.7 ± 0.4	1–2.5
hBRM (SMARCA2)	NM_003070	3.1 ± 2.0	1–9.3
SNF5 (SMARCB1)	NM_003073	3.1 ± 1.0	1–4.4
BAF60b (SMARCD2)	NM_003077	5.4 ± 4.9	1–8.9
Corepressors			
NCOR1	NM_006311	5.7 ± 3.4	1–15.5
SMRT (NCOR2)	NM_006312	2.9 ± 1.2	1–5.2
REA	NM_007273	4.5 ± 2.6	1–12.2
HDAC1	NM_004964	2.1 ± 0.7	1–3.2
HDAC2	NM_001527	2.9 ± 2.4	1–11.1
HDAC3	NM_003883	2.1 ± 1.2	1–6.4
SIN3B (KIAA0700)	XM_050561	2.2 ± 1.4	1–6.8
SAP18	NM_005870	2.4 ± 1.2	1–5.5
SAP30	NM_003864	2.2 ± 1.5	1–7.0
MTA1	NM_004689	2.2 ± 1.0	1–4.9
MTA1L1	NM_004739	6.2 ± 3.9	1–14.5

[a] Bold characters: coregulator genes selected to be additionally studied in the series of 99 ERα-positive breast tumors.

onistic effect of tamoxifen for the ERα ligand-binding site, the main mechanism underlying the antiproliferative activity of this drug (8), tamoxifen could also inhibit cell proliferation by promoting apoptosis, as shown *in vitro* and *in vivo* (9, 10). Tamoxifen-induced apoptosis may be associated with the recruitment of coregulators, which do not normally interact with estrogen-ERα complexes, leading to abnormal transcriptional regulation of ERα target genes (11).

To identify the ERα coregulators involved in tamoxifen resistance, we used real-time quantitative RT-PCR assays to analyze the expression of a large panel of coregulators (16 coactivators and 11 corepressors; Table 1) in 14 ERα-positive breast tumors. We then additionally investigated five coregulators of interest, comprising three coactivators, *TIF2* (12), *AIB1* (13), and *GCN5L2* (14) and two corepressors, *NCOR1* (15) and *MTA1L1* (16), in a well-characterized series of 99 ERα-positive unilateral invasive primary breast tumors from postmenopausal patients who exclusively received tamoxifen as adjuvant hormone therapy after primary surgery. Relationships between the mRNA levels of these coregulators, and clinical and pathological parameters, including RFS, were then studied. Finally, we sought relationships between mRNA levels of the coregulators and those of well-known molecular markers in breast cancer (*ER*α, *ER*β, and *PR*), and two candidate predictors of the response to endocrine therapy, *CCND1* (17) and *ERBB2* (18), of which the expression has been shown recently to be regulated by ERα coactivator genes (19, 20).

Table 2 Characteristics of the 99 postmenopausal patients with ERα-positive breast tumors and relation to RFS

	RFS		
	Number of patients	Number of events[a] (%)	P^b
Age			NS[c]
≤70	50	20 (40.0)	
>70	49	13 (26.5)	
Histological grade[d]			0.0012
I	13	2 (15.4)	
II	64	17 (26.6)	
III	21	13 (61.9)	
Lymph node status			0.0011
0	16	2 (12.5)	
1–3	56	15 (26.8)	
>3	27	16 (59.3)	
Macroscopic tumor size[e]			0.015
≤30 mm	67	18 (26.9)	
>30 mm	30	14 (46.7)	

[a] First relapses (local and/or regional recurrences, and/or metastases).
[b] *P* (log-rank test).
[c] NS, not significant.
[d] Scarff Bloom Richardson classification. Information available for 98 patients.
[e] Information available for 97 patients.

Materials and Methods

Patients and Samples

We analyzed samples of primary breast tumors excised from 99 women at Centre René Huguenin from 1980 to 1994. The samples were examined histologically for the presence of tumor cells. A tumor sample was considered suitable for this study if the proportion of tumor cells was >60%. Immediately after surgery, the tumor samples were placed in liquid nitrogen until total RNA extraction.

The patients (mean age, 70.7 years; range, 54–86) met the following criteria: primary unilateral ERα-positive nonmetastatic postmenopausal breast carcinoma; complete clinical, histological, and biological information available; no radiotherapy or chemotherapy before surgery; and full follow-up at Centre René Huguenin. The histological type and the number of positive axillary nodes were established at the time of surgery. The malignancy of infiltrating carcinomas was scored according to the histoprognostic system of Bloom and Richardson (21). ERα-positive status was determined at the protein level by using biochemical methods (dextran-coated charcoal method until 1988 and enzymatic immuno-assay thereafter) and confirmed by ERα real-time quantitative RT-PCR assay. Standard prognostic factors are reported in Table 2. Thirty patients had modified radical mastectomy, and 69 had breast-conserving surgery plus locoregional radiotherapy. The patients underwent physical examinations and routine chest radiography every 3 months for 2 years, then annually. Mammograms were done annually. The median follow-up was 6 years (range, 1.5–17.5 years). All of the patients received postoperative adjuvant endocrine therapy (tamoxifen, 20 mg daily for 3–5 years) and no other treatment. Thirty-three patients relapsed. The first relapse events consisted of local and/or regional recurrences in 3 patients, metastases in 26 patients, and both events in 4 patients.

Table 3 Oligonucleotide primer sequences

Gene	Oligonucleotide	Sequence	PCR product size (bp)
TIF2	Upper primer	5′-GCT GGG AGG ACC TGG TAA GAA-3′	117
	Lower primer	5′-TGA ATG CCA ATC CTT GTC TCA G-3′	
AIB1	Upper primer	5′-GAC CGC TTT TAC TTC AGG CAT T-3′	125
	Lower primer	5′-TGT GTT AAC CAG GTC CTC TTG CT-3′	
GCN5L2	Upper primer	5′-CTT CAG TCA GTG CAG CGG TTG-3′	123
	Lower primer	5′-TCC TCT TCT CGC CTG GCA TAG-3′	
NCOR1	Upper primer	5′-CCC AGC AAC GAG AGG AAT CA-3′	91
	Lower primer	5′-GTC CAT GGG AGG AGT GCT TGT-3′	
MTA1L1	Upper primer	5′-CCG ACG GCC TTA TGC TCC T-3′	145
	Lower primer	5′-CTG GGC CAC CAG ATC TTT GAC-3′	
TBP	Upper primer	5′-TGC ACA GGA GCC AAG AGT GAA-3′	132
	Lower primer	5′-CAC ATC ACA GCT CCC CAC CA-3′	

mRNA expression of the 27 coregulators was first determined in 14 ERα-positive samples from among the 99 postmenopausal breast tumors.

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 higher the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The parameter Ct 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 precise amount of total RNA added to each reaction mix (based on absorbance) and its quality (lack of extensive degradation) are both difficult to assess. Therefore, we also quantified transcripts of the gene coding for the TBP (a component of the DNA-binding protein complex TFIID) as the endogenous RNA control and normalized each sample on the basis of its TBP content (22).

The relative target gene expression level was also normalized to the expression in the breast tumor sample (calibrator) from the two series ($n = 14$ or $n = 99$), which contained the smallest amount of target gene mRNA.

Final results, expressed as N-fold differences in target gene expression relative to the *TPB* gene and the calibrator, and termed "N target," were determined as follows: N target = $2^{(\Delta Ct\ calibrator\ -\ \Delta Ct\ sample)}$, where ΔCt values of the calibrator and sample are determined by subtracting the Ct value of the target gene from the Ct value of the TBP gene.

Primers and PCR Consumables. The primers for the chosen coregulator genes and *TBP* were chosen with the assistance of Oligo 4.0 software (National Biosciences, Plymouth, MN). We conducted BLASTN searches against dbEST and nr (the nonredundant set of the GenBank, EMBL, and DDBJ database sequences) to confirm the total gene specificity of the chosen nucleotide sequences and the absence of DNA polymorphisms. The primer nucleotide sequences for the 5 coregulator genes (and *TBP*) analyzed in the full panel of 99 breast tumors are shown in Table 3; the other coregulator gene primer sequences are available on request. The primer sequences for *ERα*, *ERβ*, *PR*, *ERBB2*, and *CCND1* have been published elsewhere (22–24). To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or in a different exon. For example, the upper primer of *TBP* was placed at the junction between exon 5 and 6, and the lower primer in exon 6.

RNA Extraction. Total RNA was extracted from tissue specimens by using the acid-phenol guanidium method (25). The quality of RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; the 18S and 28S RNA bands were visualized under UV light.

cDNA Synthesis. RNA was reverse-transcribed in a final volume of 20 μl containing 1× RT-PCR buffer [500 mM each deoxynucleotide triphosphate, 3 mM $MgCl_2$, 75 mM KCl, and 50 mM Tris-HCl (pH 8.3)], 10 units of RNasin RNase inhibitor (Promega, Madison, WI), 10 mM DTT, 50 units of Superscript II RNase H^- reverse transcriptase (Life Technologies, Inc.), 1.5 mM random hexamers (Pharmacia, Uppsala, Sweden), and 1 μg of total RNA (patient samples). Samples were incubated at 20°C for 10 min and 42°C for 30 min, and reverse transcriptase was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min.

PCR Amplification. All of the PCR reactions were performed using a ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA).

For each PCR run, a master mix was prepared on ice with 1× SYBR Green buffer, 5 mM $MgCl_2$, 200 μM dATP, dCTP, and dGTP, and 400 μM dUTP, 300 nM each primer, and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems). Five μl of each diluted cDNA solution sample was added to 20 μl of the PCR master-mix. 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 65°C for 1 min.

Statistical Analysis

RFS was determined as the interval between initial diagnosis and detection of the first relapse (local and/or regional recurrence, and/or metastasis). Mean ERα coregulator mRNA levels were compared by using the Kruskal-Wallis test. Spearman's rank correlation test was used to study relationships between continuous variables. Survival distributions were esti-

Table 4 Mean, median, and range of coregulator genes mRNA levels in 99 ERα-positive breast tumors

	Coactivators			Corepressors	
	TIF2	*AIB1*	*GCN5L2*	*NCOR1*	*MTA1L1*
Mean ± SD	7.7 ± 4.5	2.6 ± 1.5	6.9 ± 5.3	5.6 ± 2.9	4.3 ± 2.0
Median	6.5	2.3	5.1	5.2	4.1
Range	1–31.3	1–12.2	1–35.3	1–16.9	1–18.5

Table 5 Relationships between coregulator genes mRNA levels in 99 ERα-positive breast tumors

	Coactivators			Corepressors
	TIF2	*AIB1*	*GCN5L2*	*NCOR1*
AIB1	+0.346[a] 0.00057[b]			
GCN5L2	+0.298 0.0029	+0.220 0.027		
NCOR1	+0.371 0.00022	+0.073 NS[c]	+0.398 0.000077	
MTA1L1	+0.240 0.016	+0.378 0.00017	+0.174 NS (0.080)	+0.225 0.024

[a] Spearman correlation coefficient.
[b] *P*, Spearman rank correlation test.
[c] NS, not significant.

mated by using the Kaplan-Meier method (26), and the significance of differences between survival rates was determined using the log-rank test. Multivariate analysis using Cox's proportional hazards model was used to assess the independent contribution of each variable to RFS (27). Differences between groups were judged significant at confidence levels >95% ($P < 0.05$).

Results

Expression Analysis of 27 Coregulator Genes in 14 ERα-Positive Breast Tumors

We measured mRNA expression levels of 27 ERα coregulator genes in a series of 14 ERα-positive breast tumor samples (Table 1). The widest ranges of values for a coactivator gene and a corepressor gene were, respectively, from 1 to 17.6 for *TIF2* and from 1 to 15.5 for *NCOR1*. We then additionally studied selected genes in a larger series of 99 ERα-positive breast tumors. The selection was based on: (*a*) the widest range of expression in the initial series of 14 breast tumor samples; (*b*) selection of two genes from each coactivator and corepressor type of genes; and (*c*) coregulator genes reported previously to be dysregulated in breast cancer (*AIB1*; Ref. 13).

Thus, we selected two coactivators that interact directly with ERα (*TIF2* and *AIB1*), a coactivator involved in nucleosome disruption (*GCN5L2*), a corepressor that interacts with ERα (*NCOR1*), and a corepressor belonging to a histone deacetylase complex (*MTA1L1*).

Expression Analysis of Five Coregulator Genes in 99 Postmenopausal ERα-Positive Breast Tumors

Coregulator mRNA Steady-State Levels in Breast Tumor Samples. Table 4 shows the mean, median, and range of *TIF2, AIB1, GCN5L2, NCOR1,* and *MTA1L1* mRNA levels in the 99 postmenopausal ERα-positive breast tumors. The range of *TIF2* and *GCN5L2* expression exceeded 30-fold, whereas that of the other three coregulators did not exceed 20-fold.

Relationships among Coregulator mRNA Levels. We then sought links between mRNA values for each of the coregulators by using the Spearman rank correlation test (Table 5). Among the coactivators, *TIF2* mRNA levels correlated with both *AIB1* and *GCN5L2* mRNA levels ($P = 0.00057$, $r = +0.346$, and $P = 0.0029$, $r = +0.298$, respectively). A positive correlation was also found between *AIB1* and *GCN5L2* mRNA levels ($P = 0.027$, $r = +0.220$). Regarding the two corepressors, a weak positive correlation was observed between their mRNA levels ($P = 0.024$, $r = +0.225$). The following strong positive correlations were observed between coactivators and corepressors: *AIB1* (coactivator) and *MTA1L1* (corepressor; $P = 0.00017$, $r = +0.378$), *TIF2* and *NCOR1* ($P = 0.00022$, $r = +0.371$), and *GCN5L2* and *NCOR1* ($P = 0.000077$, $r = +0.398$). A weak positive correlation was also found between *TIF2* and *MTA1L1* mRNA levels ($P = 0.016$, $r = +0.240$).

Relationships between Coregulator mRNA Levels, and Clinical and Pathological Parameters. Coregulator mRNA levels were compared among patient subgroups defined by clinical and pathological parameters (age, lymph node status, histological grade, and macroscopic tumor size) using the Kruskal-Wallis test (Table 6). No difference in mean coregulator mRNA levels was found.

Relationships between Coregulator and *ERα*, *ERβ*, *PR*, *CCND1*, and *ERBB2* mRNA Levels. We then looked for relationships between mRNA levels of coregulators and those of classical molecular markers, *i.e., ER*α, *ER*β, *PR, CCND1,* and *ERBB2*, determined previously using the same methodology (Table 6; Ref. 28).[4] A positive correlation was only observed between *ER*α and the coactivator *GCN5L2* (Spearman rank test; $P = 0.015$, $r = +0.243$). A trend toward statistical significance was observed between mRNA levels of *ER*α and *NCOR1*, a corepressor ($P = 0.061$, $r = +0.187$). No correlation was observed between coregulator and *ER*β mRNA levels. mRNA levels of *PR* (a well-known ERα-responsive gene) correlated positively with those of *TIF2* ($P = 0.0083$, $r = +0.263$) and *NCOR1* ($P = 0.0011$, $r = +0.328$), and a trend toward significance was observed with *GCN5L2* ($P = 0.054$, $r = +0.192$). Highly significant positive correlations were found between mRNA levels of *CCND1* and four coregulators, *AIB1* only showing a trend toward significance ($P = 0.10$). The only significant correlation found for *ERBB2* was with *AIB1* ($P = 0.011$, $r = +0.254$).

Prognostic Value of Coregulator mRNA Levels. For the prognostic analysis of each gene, the patient population was divided into tertiles (three groups of 33), corresponding to low, intermediate, and high mRNA levels. Only *NCOR1* showed

[4] F. Spyratos, M. Labroquère, M. Tubiana-Hulin, S. Tozlu, M. Vidaud, K. Hacène, R. Lidereau, V. Becette, and I. Bièche. Expression of estrogen receptor beta mRNA in postmenopausal primary breast cancer patients receiving adjuvant tamoxifen, submitted for publication.

Table 6 Relationships between coregulator genes mRNA levels and clinical, pathological, and molecular parameters in ERα-positive breast tumors of 99 postmenopausal patients

		Coactivators						Corepressors			
		TIF2		*AIB1*		*GCN5L2*		*NCOR1*		*MTA1L1*	
Age			NS[a]		NS		NS		NS		NS
≤70	50[b]	8.3 ± 5.4[c]		3.7 ± 2.3		6.7 ± 5.4		5.8 ± 3.4		4.3 ± 2.1	
>70	49	7.0 ± 3.3		3.4 ± 1.7		7.1 ± 5.3		5.4 ± 2.3		4.0 ± 1.8	
Histological grade[d]			NS		NS		NS		NS		NS
I	13	8.1 ± 3.8		2.7 ± 0.9		5.4 ± 1.8		6.1 ± 2.6		4.3 ± 1.6	
II	64	8.0 ± 4.6		3.8 ± 2.3		7.2 ± 5.6		5.6 ± 2.6		4.1 ± 2.0	
III	21	6.7 ± 4.5		3.3 ± 1.4		6.6 ± 5.8		5.3 ± 4.0		3.8 ± 1.6	
Lymph node status			NS		NS		NS		NS		NS
0	16	8.1 ± 6.7		3.2 ± 1.6		7.6 ± 5.9		5.0 ± 2.2		3.6 ± 1.6	
1–3	56	7.5 ± 3.5		3.4 ± 2.2		5.4 ± 6.9		6.0 ± 2.9		4.3 ± 2.1	
>3	27	7.9 ± 4.7		4.0 ± 1.9		4.9 ± 6.8		5.3 ± 3.3		4.1 ± 1.8	
Macroscopic tumor size[e]			NS		NS		NS		NS		NS
≤30 mm	67	8.0 ± 4.7		3.6 ± 2.1		6.4 ± 4.2		5.7 ± 2.7		3.8 ± 1.4	
>30 mm	30	6.9 ± 4.0		3.4 ± 1.9		7.5 ± 7.2		5.2 ± 3.1		4.7 ± 2.6	
mRNA levels											
ERα	99	+0.125[f]	NS	−0.075	NS	+0.243	0.015	+0.187	NS (0.06)	+0.008	NS
ERβ	99	−0.047	NS	+0.153	NS	+0.109	NS	+0.170	NS (0.09)	+0.170	NS (0.09)
PR	99	+0.263	0.0083	−0.002	NS	+0.192	NS (0.05)	+0.328	0.0011	+0.135	NS
CCND1	99	+0.328	0.0011	+0.162	NS (0.10)	+0.258	0.0097	+0.272	0.0064	+0.295	0.0031
ERBB2	99	+0.020	NS	+0.254	0.011	+0.153	NS (0.13)	−0.109	NS	+0.05	NS

[a] *P*, Kruskal-Wallis test for clinical and pathological parameters and Spearman rank correlation test for molecular mRNA levels.
[b] Case number.
[c] Mean mRNA levels ± SD.
[d] Scarff Bloom Richardson classification. Information available for 98 patients.
[e] Information available for 97 patients.
[f] Spearman correlation coefficient.

prognostic value. Five-year RFS rates were 58.7% (49.8–67.6%) in the subgroup of low *NCOR1* mRNA levels, and 80.8% (73.7–87.9%) and 83.1% (76.1–90.1%) in the subgroups with intermediate and high mRNA levels, respectively. Given the similar values in the subgroups with intermediate and high *NCOR1* mRNA levels, we compared full follow-up RFS between the low *NCOR1* subgroup (33 patients) and the combined intermediate and high subgroups (66 patients). Univariate analysis (log-rank test) showed that the outcome of the former 33 patients was significantly worse than that of the remaining 66 patients ($P = 0.0076$; Fig. 1).

Using a Cox proportional hazards model, we then assessed the prognostic significance of the four parameters that were significant in univariate analysis (histopathological grade, lymph node status, macroscopic tumor size, and *NCOR1* status; Table 2; Fig. 1). The prognostic significance of histopathological grade, lymph node status, and *NCOR1* status persisted, whereas that of macroscopic tumor size disappeared (Table 7).

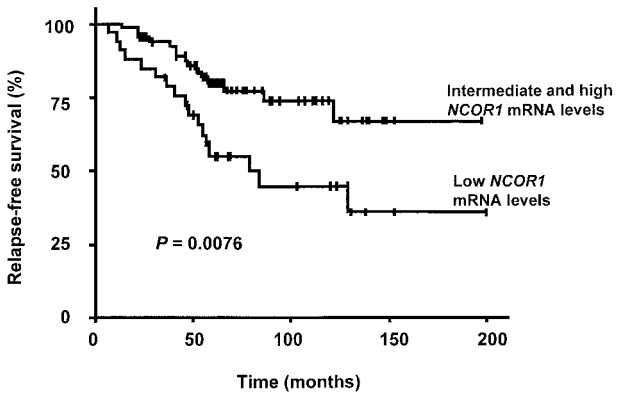

Fig. 1 RFS in the patient subgroup with low *NCOR1* mRNA levels (33 patients), and the combined subgroups with intermediate and high *NCOR1* mRNA levels (66 patients).

Discussion

To gain more insight into the roles of the different ERα coregulator genes in breast tumors, we have chosen a two-step strategy to measure mRNA expression of coregulator genes by using real-time quantitative RT-PCR. The first step was aimed to quantify the mRNA expression of a large panel of genes (n = 27) coding for coregulators in a small homogeneous series of 14 ERα-positive breast tumors and, thus, to distinguish expressed from unexpressed coregulators in breast tumors. Given the high number of coregulator genes, it is suspected that related genes may show functional redundancy (29). Therefore, in a second step, in addition to *AIB1* (reported previously as being dysregulated in breast cancer; Ref. 13), two other coactivators (*TIF2* and

Table 7 Multivariate analysis of relapse-free survival (RFS)

	RFS		
	Regression coefficient	Relative risk (95% CI)[a]	*P*
Histological grade	0.83		0.0098
I		1.0	
II		2.3 (1.2–4.3)	
III		5.2 (1.5–18.4)	
Lymph node status	0.83		0.0078
0		1.0	
1–3		2.3 (1.2–4.2)	
>3		5.2 (1.5–17.6)	
Macroscopic tumor size	0.44		0.24
≤30 mm		1.0	
>30 mm		1.6 (0.8–3.2)	
NCOR1 mRNA levels	0.75		0.043
Low		2.1 (1.0–4.3)	
Intermediate and high		1.0	

[a] 95% confidence interval.

GCN5L2) and two corepressors (*NCOR1* and *MTA1L1*) were selected for their wide range of mRNA expression levels (>10-fold), and were additionally analyzed in a well-characterized series of 99 ERα-positive breast tumors from postmenopausal patients treated exclusively with tamoxifen after surgery.

mRNA levels of the three coactivators correlated positively with one another as well as mRNA levels of the two corepressor genes (Table 5). Positive correlations were also observed in all of the pairwise comparisons of the coactivators and corepressors, except for *AIB1* with *NCOR1* and *GCN5L2* with *MTA1L1*. These correlations may be explained by: (*a*) transcriptional activation of all five of the genes by a common regulatory pathway; (*b*) specific up-regulation of one coactivator directly involved in transcriptional activation of the correlated coactivator and/or corepressor; and (*c*) up-regulation of corepressors aimed at controlling coactivators overexpression.

With respect to clinical and pathological parameters, ERα coregulator expression was unrelated to age, histological grade, lymph node status, and macroscopic tumor size (Table 6). Previous studies of breast tumors have shown a significant relationship between *AIB1* overexpression and high tumor grade (30), and also higher *TIF2* expression levels in node-positive than node-negative breast tumors (31). These discrepancies with our study could be explained in large part by differences in the study populations. Indeed, we studied a well-defined series of postmenopausal patients with ERα-positive tumors.

The only significant relationship between ERα coregulator gene expression, and *ER*α and *ER*β expression was a positive correlation between *GCN5L2* and *ER*α, suggesting an interaction between the transcriptional regulation of the two genes, despite the lack of a consensus ERE sequence in the promoter region of the *GCN5L2* gene (data not shown). Expression of the *TIF2* coactivator and the *NCOR1* corepressor was significantly linked to expression of the PR gene, which contains an ERE element in its promoter, suggesting a predominant role of these two genes in the transcriptional regulation of ERα-responsive genes mediated by ERE.

It has been shown recently that transcriptional regulation of two candidate predictors of the response to endocrine therapy, the genes *CCND1* and *ERBB2*, can be exerted by ERα coactivators, despite the lack of an ERE in the promoter region of the two genes (19, 20). *AIB1* was found to enhance *CCND1* expression when incubated with estrogen, whereas antiestrogen treatment had no activating or inhibiting effect on *CCND1* transcription. In our study, significant positive correlations were observed between mRNA expression of *CCND1* and that of all of the coregulators but only a trend toward significance with *AIB1*, suggesting that *AIB1* is not a major coactivator involved in *CCND1* transcriptional regulation during breast tumorigenesis. Regarding the *ERBB2* gene, Newman *et al.* (20) showed that p160 coactivators (of which AIB1 is a member) can modulate its enhancer activity. In our series, *ERBB2* mRNA levels correlated significantly with *AIB1* mRNA levels but not with the other four ERα coregulators. The same relationship was reported at the protein level by Bouras *et al.* (30), who found that tumors overexpressing AIB1 showed strong ERBB2-positive staining. These results suggest that transcriptional coactivators may establish cross-talk between ERE-dependent and -independent metabolic pathways involved in breast tumorigenesis.

The breast tumor samples studied here were obtained from a well-characterized series of patients treated exclusively with tamoxifen as adjuvant endocrine therapy. Therefore, we were able to evaluate the potential of the five selected ERα coregulator genes as predictors of tamoxifen resistance. The hypothesis of *NCOR1* being a potential marker of antiestrogen hormonotherapy (32) is conceivably related to *in vitro* studies that have established that NCOR1 protein binds ERα (whether or not the interaction is tamoxifen-dependent is controversial; Refs. 33–35), and inhibits the partial agonistic activity of tamoxifen and its physiological metabolite (4-hydoxy-tamoxifen; Refs. 35, 36). Likewise, in a mouse model of breast cancer, decreased *NCOR1* protein expression correlated with acquired tamoxifen resistance (33).

In human breast tumors, Chan *et al.* (37) did not show significant difference in the level of *SMRT* mRNA (a corepressor related to *NCOR1*) between tamoxifen-resistant breast tumor samples and untreated patients. Conversely, Kurebayashi *et al.* (31) found higher *NCOR1* expression levels in tumors from patients without recurrence compared with patients with recurrence supporting its role as a tamoxifen resistance mechanism *in vivo*.

Here, we found that *NCOR1* mRNA expression status (low *versus* intermediate and high) was associated with shorter RFS (log-rank test; $P = 0.0076$), and this prognostic significance persisted in Cox multivariate regression analysis ($P = 0.043$). Nevertheless, validation of the predictive value of *NCOR1* parameter in the response to tamoxifen endocrine therapy in breast cancer patients needs a prospective randomized study to show that this parameter does influence the outcome only in patients who received adjuvant tamoxifen compared with untreated patients.

We showed previously, in the same patients series, that *ERBB2* overexpression was a marker of poor prognosis (28). By combining *NCOR1* and *ERBB2* expression status, we identified four separate prognostic groups. The patients with the best prognosis had high *NCOR1* expression and normal *ERBB2* expression (log-rank test; $P = 0.00064$), suggesting that the analysis of the two molecular markers together, affecting ta-

moxifen efficiency, may provide a more accurate prediction of hormone responsiveness (38).

In conclusion, *NCOR1* emerged from a series of 27 ERα coregulator genes as a promising independent prognostic marker of tamoxifen resistance in patients with ERα-positive breast tumors. Therefore, *NCOR1* real-time RT-PCR assay should be valuable in the breast cancer clinical setting to better assess endocrine treatment efficiency.

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