Identification of *TACC1*, *NOV*, and *PTTG1* as new candidate genes associated with endocrine therapy resistance in breast cancer

Sandra E Ghayad^{1,2,3,4*}, Julie A Vendrell^{1,2,3,4*}, Ivan Bieche⁵, Frédérique Spyrtatos⁵, Charles Dumontet^{1,3,6}, Isabelle Treilleux⁴, Rosette Lidereau⁵ and Pascale A Cohen^{1,2,3,4}

¹Université de Lyon, 69008 Lyon, France,

²Université Lyon 1, ISPB, Faculté de Pharmacie de Lyon, 69008 Lyon, France,

³INSERM, U590, 69008 Lyon, France,

⁴Centre Léon Bérard, FNCLCC, 69008 Lyon, France,

⁵Laboratoire d'Oncogénétique, INSERM U735, 92210 St-Cloud, France

⁶Université Lyon 1, Faculté de Médecine, 69008 Lyon, France

(Correspondence should be addressed to P A Cohen; Email: pascale.cohen@recherche.univ-lyon1.fr)

* (S E Ghayad and J A Vendrell contributed equally to this work)

Abstract

Cross-resistance to molecules used in endocrine therapy is among the main challenges in the treatment of estrogen receptor- α (ER α) positive breast cancer. In this study, we used two different cell models of resistance to anti-estrogens: MVLN/CL6.7 cells and VP229/VP267 cells selected after exposure to tamoxifen respectively *in vitro* and *in vivo* to characterize a phenotype rarely observed, i.e. acquisition of cross-resistance to the pure ER antagonist fulvestrant. As MVLN/CL6.7 cells and VP229/VP267 cell lines are original and valuable models of cross-resistance to tamoxifen and fulvestrant, we examined candidate genes using a RTQ-PCR strategy to identify new biomarkers of endocrine resistance. Out of the 26 candidate genes tested, 19 displayed deregulation of expression at the basal level in at least one of the two resistant cell lines. Eight genes (*TACC1*, *NOV*, *PTTG1*, *MAD2L1*, *BAK1*, *TGFB2*, *BIRC5*, and *CCNE2*) were significantly overexpressed in samples from ER-positive breast cancer patients who relapsed after tamoxifen treatment ($n=24$) compared with samples from patients who did not ($n=24$). Five genes (*TACC1*, *NOV*, *PTTG1*, *BAK1*, and *TGFB2*) were correlated with significantly shorter relapse-free survival (univariate analysis). Finally, we identified *TACC1* and a three-gene expression signature (*TACC1*, *NOV*, and *PTTG1*) as independent prognostic markers (multivariate analysis). Aberrant mRNA and protein levels of *TACC1*, *NOV*, and *PTTG1* were also observed under tamoxifen and/or fulvestrant exposure in resistant CL6.7 cells compared with their respective control MVLN cells. In conclusion, our data identify *TACC1*, *NOV*, and *PTTG1* as promising new markers that could be used in the clinical management of ER-positive breast cancer patients.

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Introduction

Breast carcinoma, the most common female cancer, is currently undergoing an alarming increase. Breast cancer growth is regulated by estrogen, which acts by binding to the estrogen receptor α (ER α). ER α is routinely used as a prognostic and predictive marker in the management of breast cancer patients, in particular to identify patients who may respond to endocrine therapy. The selective estrogen receptor modulator tamoxifen (Tam) which aims to block the ER α has been the mainstay of hormonal therapy in both early and advanced breast cancer patients for approximately three decades (EBCTC Group 1998, Gradishar 2005). However, Tam is only partially effective because of intrinsic or acquired tumor resistance. Approximately, 40% of patients with

ER-positive breast cancer will not respond to Tam (*de novo* resistance). Moreover, long-term follow-up and clinical trials have demonstrated that up to 62% of cancers initially responsive to endocrine therapy subsequently escape control with the patient requiring salvage surgery (Horobin *et al.* 1991, Fennessy *et al.* 2004). In general, Tam-resistant tumors respond to second-line endocrine therapy, suggesting that resistance is not attributable to the loss of or alterations in ER α , and that tumor growth may involve functional ER α signaling (Howell *et al.* 1995, Osborne 1998). New endocrine therapies have recently emerged, in particular with the selective estrogen receptor down-regulator (SERD) fulvestrant (ICI 182,780). The mechanism of action of this ER antagonist is different from that of Tam, as binding of fulvestrant to ER induces destabilization and degradation of the

receptor. Moreover, Tam only blocks the transcriptional activating function AF2 of ER, and thus only partially inactivates ER-regulated transcription (Tzakerman *et al.* 1994, McDonnell *et al.* 1995), while fulvestrant blocks both AF1 and AF2 functions of ER, resulting in complete abrogation of the transcription of ER-regulated genes (Wakeling *et al.* 1991, Dowsett *et al.* 2005). Two phase III trials have demonstrated that fulvestrant is as efficient as anastrozole, a third-generation aromatase inhibitor, in second-line treatment for postmenopausal women with advanced-stage Tam-resistant breast cancer (Dodwell & Pippen 2006). Recent clinical trials have also shown that in patients with advanced breast cancer progressing on prior endocrine therapy with Tam or an aromatase inhibitor, fulvestrant produces a clinical benefit in respectively 43% and 30% of the patients (Howell *et al.* 2002, Osborne *et al.* 2002, Pery *et al.* 2007). These clinical data underline not only the importance of fulvestrant in the therapeutic arsenal against ER+ breast cancers, but also suggest that in a subset of patients who have developed resistance to first-line endocrine therapy (with Tam or aromatase inhibitors), cross-resistance to fulvestrant can also occur.

In *in vitro*-established resistant breast cancer cells, Tam-resistant cells selected under Tam exposure are usually sensitive to fulvestrant (Parisot *et al.* 1999, Kilker *et al.* 2004, Martin *et al.* 2005, Shaw *et al.* 2006), whereas resistant cell lines established under fulvestrant selection are commonly cross-resistant to Tam (Lykkesfeldt *et al.* 1995, Brunner *et al.* 1997, Fan *et al.* 2006a, Shaw *et al.* 2006). Few studies have reported a decreased sensitivity of Tam-resistant breast cancer cells or xenografts to fulvestrant (Lykkesfeldt *et al.* 1994, Naundorf *et al.* 2000), but none, to our knowledge, has demonstrated the total abrogation of fulvestrant response. In this study, we fully characterized for the first time the endocrine resistance developed by two cellular models of acquired Tam resistance: *in vitro*-selected Tam-resistant CL6.7 cells (Badia *et al.* 2000) and *in vivo*-selected Tam-resistant VP267 cells (McCallum & Lowther 1996). Strikingly, we found that, while selected under Tam exposure, CL6.7 and VP267 cells also acquired cross-resistance to fulvestrant, characterized by a total abrogation of the SERD's response. Thus, the combination of CL6.7 and VP267 cells represents a good cellular model for the genomic screening of molecular markers of endocrine resistance with potential clinical relevance. The recent development of effective tools for investigating gene-expression profiles has already led to the identification of new diagnostic and prognostic markers. These tools include cDNA arrays (van 't Veer *et al.* 2002, van de Vijver *et al.* 2002, Ma *et al.* 2004, Frasor *et al.* 2006) and real-time quantitative RT-PCR (RTQ-PCR). While cDNA array allows the screening of a large number of genes at a

time, RTQ-PCR is an alternative approach that has already proved useful for the identification of molecular markers. Indeed, RTQ-PCR is an accurate, sensitive and reproducible technique more suitable for detecting small variations in gene expression and weakly expressed genes (Bieche *et al.* 2004). While this technique allows the quantitative measurement of only a small number of selected candidate genes, it has already been successfully used in several studies aimed at identifying markers associated with endocrine therapy resistance (Bieche *et al.* 2004, Paik *et al.* 2004, Tozlu *et al.* 2006).

In two previous genomic studies, we had identified by cDNA arrays new genes demonstrated as estrogen-modulated or associated with agonist activity of 4-hydroxy-tamoxifen (OH-Tam) and hyper-response to 17 β -estradiol (E_2) in OH-Tam-resistant breast cancer cell lines (Vendrell *et al.* 2004, 2005). With the aim to identify gene expression deregulation associated with resistance to Tam and fulvestrant, we selected from these studies 26 candidate genes whose biological functions are associated with cell proliferation, transformation, apoptosis, DNA repair, and DNA replication. We analyzed by RTQ-PCR the expression of the 26 candidate genes in CL6.7 and VP267 cells. Genes with a deregulated expression in resistant cells were then selected to be explored in a series of 48 ER+ breast tumor samples from postmenopausal patients, who relapsed after primary surgery and adjuvant endocrine therapy ($n=24$) or did not ($n=24$).

Materials and methods

Cell-proliferation analysis

MVLN, CL6.7, CL6.8, VP229, and VP267 cells were grown as described previously (Demirpence *et al.* 1993, McCallum & Lowther 1996), then purged for 4 days in DMEM without Phenol Red and supplemented with 3% steroid-depleted, dextran-coated, charcoal-treated FCS (DCC medium). The cells were then treated (with media changed every two days) in the absence or the presence of E_2 (Sigma, Saint Louis, MO, USA), OH-Tam (Sigma) or fulvestrant (Tocris, Ellisville, MO, USA). Proliferating cells were analyzed using the cell proliferation ELISA, 5-bromodeoxyuridine (BrdU) colorimetric kit (Roche) as previously described (Vendrell *et al.* 2005).

Western blot

Cell extract preparation and western blot analysis were performed as previously described (Vendrell *et al.* 2004). ER α and NOV antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA),

TACC1 antibody from Upstate (Lake Placid, NY, USA), PTTG1 antibody from Zymed Laboratories (San Francisco, CA, USA) and α -tubulin antibody from Sigma.

Breast tumor samples

Samples from primary breast tumors were obtained from women operated on at the Centre René Huguenin (St Cloud, France) between 1980 and 1994. Informed consent was obtained from all patients and the study was approved by the ethics committee of the institution. A set of 48 ER+ breast tumor samples was used in this study; the tumors were taken from patients treated with primary surgery followed by adjuvant Tam alone: thirty-nine (81.2%) underwent a mastectomy and nine (18.8%) had breast-conserving surgery. Twenty-four patients relapsed under Tam treatment and developed metastases ('Relapse' group) and 24 patients did not ('No relapse' group). Standard prognostic factors are shown in Table 1. The patients (mean age: 70.7 years; range: 54–86) met the following criteria: primary unilateral postmenopausal breast carcinoma; complete clinical, histological, and biological information available; no radiotherapy or chemotherapy before surgery; and full follow-up at the 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 histological grading system of Bloom & Richardson (1957) and Elston & Ellis (1993). At the time of surgery (years 1980–1994), ER α status was determined at the protein level by using quantitative biochemical methods: dextran-coated charcoal method until 1988 and enzymatic immunoassay

thereafter. We have recently confirmed the ER α status of these 48 tumors by RTQ-PCR. The patients had physical examinations and routine chest radiography every 3 months for 2 years, then annually. Mammograms were performed annually. The median follow-up was 7.2 years (range 1.5–10.0 years). All the patients received post-operative adjuvant endocrine therapy (Tam, 20 mg daily for 3–5 years), and no other treatment. Patients in the 'Relapse' group experienced metastases between 1.3 and 10.0 years after surgery and the beginning of Tam treatment. Tumor samples containing more than 70% of tumor cells were considered suitable for the study. Immediately following surgery, the tumor samples were placed in liquid nitrogen until RNA extraction. Specimens of adjacent normal breast tissue from three breast cancer patients and normal breast tissue from three women undergoing cosmetic breast surgery were used as sources of normal RNA.

RTQ-PCR analysis

Total RNA was extracted by cesium chloride ultracentrifugation, and RNA integrity was checked using the BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). One microgram of total RNA from each sample was reverse transcribed, and RTQ-PCR measurements were performed as described previously (Girault *et al.* 2003, Vendrell *et al.* 2005) using a LightCycler (Roche) or an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with the corresponding SYBR Green Kit, according to the manufacturer's recommendations. For each gene, mRNA levels in the breast samples were normalized such that the median value of six normal human breast tissue samples was 1.

Statistical analyses

Gene-expression levels in subgroups of breast tumor samples were compared using the Kruskal–Wallis test. Differences between groups were judged significant at a confidence level greater than 95% ($P < 0.05$). To visualize the capacity of a molecular marker to discriminate between two populations (in the absence of an arbitrary cut-off value), we summarized the data in a receiver operating characteristic (ROC) curve (Hanley & McNeil 1982). ROC curves plot sensitivity (true positives) on the y-axis against one specificity (false positives) on the x-axis, considering each value as a possible cut-off. The area under curve (AUC) was calculated and used 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. Conversely, when a marker has strong discriminative value, the ROC curve comes closest to the

Table 1 Characteristics of the 48 ER+ tumors taken from patients treated with primary surgery followed by adjuvant Tam alone, and who relapsed or did not

	Number of patients	RFS ^a	
		Number of events (%)	P ^b
Age			
≤ 70	27	14 (51.9)	NS (0.39)
> 70	21	10 (47.6)	
SBR histological grade ^c			
I + II	38	16 (42.1)	NS (0.13)
III	10	8 (80.0)	
Lymph node status			
≤ 3	32	13 (40.6)	0.023
> 3	16	11 (68.7)	
Macroscopic tumor size (mm)			
≤ 30	32	14 (43.8)	NS (0.076)
> 30	16	10 (62.5)	

NS, not significant.

^aRFS, relapse-free survival. Local and/or regional recurrences, and/or metastases.

^bLog-rank test.

^cScarff–Bloom–Richardson classification.

upper left-hand corner (or to the lower right-hand corner) and the AUC is close to 1.0 (or 0). Hierarchical clustering was performed using the GenANOVA software (Didier *et al.* 2002). Relapse-free survival (RFS) was defined 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 using the log-rank test (Peto *et al.* 1977). Cox's proportional hazards regression model (Cox 1972) was used to assess prognostic significance.

Immunohistochemical and RTQ-PCR analyses of breast tumor samples

Ten ER+ frozen breast tumors from patients diagnosed at Centre Léon Bérard were obtained from the Biological Resource Centre of Centre Léon Bérard, (French agreement number: DC-2008-99) after approval by the institutional review board and ethics committee, with fully informed patient consent. Paraffin-embedded breast tumors were fixed in 10% formalin and inserted in tissue microarray blocks. The blocks containing invasive carcinoma were sectioned at a thickness of 4 µm. After deparaffinization and rehydration, endogenous peroxidases were blocked by incubating the slides in 5% hydrogen peroxide in sterile water. The slides were then incubated with a protein blocking agent (Immunotech, Marseille, France) for 10 min at room temperature. For heat-induced antigen retrieval, tissue sections were boiled using a water bath at 97 °C for 50 min either in 10 mM high pH buffer (Dako, Trappes, France) for anti-NOV antibody or in citrate buffer (Dako) for anti-PTTG1 antibody. The slides were then incubated at room temperature for 30 min with the anti-NOV goat polyclonal antibody (Santa Cruz) or for 1 h with the anti-PTTG1 polyclonal rabbit antibody (Zymed). Both antibodies were diluted using an antibody diluent solution (Dako) at 1/800 (anti-NOV antibody) or at 1/600 (anti-PTTG1 antibody). After rinsing in PBS, the slides were incubated with a biotinylated secondary antibody bound to a streptavidin–peroxidase conjugate (LSAB+ Kit, Dako). Bound antibody was revealed by adding 3,3'-diaminobenzidine. Sections were counterstained with hematoxylin. The slides were analyzed independently by the pathologist and the technician who assessed both the percentage of stained cells and the staining intensity (0 = none, 1+ = low, 2+ = intermediate, 3+ = high) in infiltrative carcinomatous cells. The tumors were then stratified according to a score established by multiplying the percentage of stained cells by the intensity. The cut-off values chosen to classify the tumors into the 'low protein expression' group or the 'high protein expression' group were determined after a pre-observation performed on a larger set of breast

tumors ($n=80$, data not shown). As three levels of intensity could be observed for NOV immunostaining and only two for PTTG1 immunostaining, the staining score had to be at least 100 for NOV and at least 40 for PTTG1 to belong to the 'high protein expression' group.

The same 10 breast tumor samples were disrupted using the TissueLyser (Qiagen) and total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. Total RNA was subsequently quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and RNA integrity was checked using the BioAnalyzer 2100 (Agilent Technologies). RTQ-PCR for *NOV* and *PTTG1* was performed as described above. For each gene, the 10 ER+ breast tumors were divided into two groups: samples with 'low mRNA expression level' (lower than the median mRNA level) and those with 'high mRNA expression level' (higher than the median mRNA level).

Results

The CL6.7 cell line developed a phenotype of OH-Tam resistance similar to that of the previously characterized CL6.8 cells

MVLN is an ER+, hormone-responsive, and OH-Tam-sensitive breast carcinoma cell line derived from MCF-7 cells (Demirpence *et al.* 1993). Six-month exposure of MVLN cells to OH-Tam allowed the emergence of several OH-Tam-resistant (OTR) but still estrogen-dependent cellular clones (Badia *et al.* 2000). We have previously fully characterized the phenotype of OTR CL6.8 cells, defined by an agonist activity of OH-Tam and hypersensitivity to E₂ (Vendrell *et al.* 2005). The OTR CL6.7 cell line remains to be characterized.

One of the mechanisms possibly involved in endocrine therapy resistance is an alteration in the expression of ERα and/or ERβ (Johnston *et al.* 1995, Speirs *et al.* 1999). We found that, as previously observed for OTR CL6.8 cells (Vendrell *et al.* 2005), ERα protein expression was decreased in OTR CL6.7 cells when compared with MVLN cells, and this decrease was more pronounced in CL6.7 than in CL6.8 cells (Fig. 1A). Investigation of *ESR1/ERα* mRNA levels by RTQ-PCR (Table 2) demonstrated no difference in the *ESR1/ERα* gene expression level between CL6.7 and MVLN cells (−1.2-fold), suggesting that the down-regulation of ERα expression in CL6.7 cells mainly occurs at a post-transcriptional level. On the contrary, *ESR1/ERα* gene expression was decreased in the CL6.8 cell line when compared with MVLN (−2.4-fold, data not shown). *ESR2/ERβ* mRNA level was 1000-fold lower than *ESR1/ERα* mRNA and no difference in expression was observed, either between OTR CL6.7 and MVLN cells

Figure 1 ER α expression and functionality in CL6.7 cells. (A) Exploration of ER α protein expression at the basal level in MVLN, CL6.7, and CL6.8 cells by western blot as described in the Materials and methods section using anti-ER α antibody. Expression of α -tubulin was used as invariant control. (B) *TFF1* (*pS2*) gene expression variations (fold change) measured by RTQ-PCR in MVLN, CL6.7, and CL6.8 cells after 4 days of E₂ treatment. (C) Proliferative response of MVLN (white bars), CL6.7 (black bars), and CL6.8 (grey bars) cells grown in DCC medium, then treated for 1, 3, 5, or 8 days with vehicle or 1 nM E₂. Results are expressed as a percentage of cells incorporating BrdU in the presence of treatment compared with untreated cells. Results are expressed as means $\pm$ s.d. from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ versus the corresponding MVLN treatment according to Student's *t*-test.

(Table 2) or between OTR CL6.8 and MVLN cells (data not shown). The functionality of the *ESR1/ER α* gene and the basal transcription machinery previously documented for CL6.8 cells (Vendrell *et al.* 2005) were also demonstrated in the present study for CL6.7 cells, based on several findings: i) 96 h exposure to E₂ (1 nM) is capable of inducing increased (+5.5-fold) *TFF1* (*pS2*) mRNA levels (Fig. 1B); ii) 24 h exposure to E₂ (1 nM) stimulates luciferase activity (+10-fold) in ERE-luciferase reporter plasmid-transfected CL6.7 cells (data not shown); iii) in a cell proliferation assay measured by BrdU labeling (Fig. 1C), E₂ is capable of stimulating CL6.7 cell proliferation. The phenotype

developed by the CL6.7 cell line also exhibits a hyper-response to E₂ evidenced by the earlier detection of E₂ stimulation in CL6.7 cells (day 3) than in the MVLN cell line (day 5), and by a significantly greater amplitude of proliferation under E₂ treatment in OTR cells than in MVLN cells (Fig. 1C), similarly to what was previously observed for CL6.8 cells (Fig. 1C and Vendrell *et al.* 2005). The phenotype of OH-Tam resistance developed by CL6.7 cells was characterized under OH-Tam exposure by the loss of the cytostatic activity of the molecule that is detectable in MVLN cells and the occurrence of a strong stimulation of CL6.7 cell proliferation (estrogen-like effect; Fig. 2A). This phenotype was very close to the one developed by OTR CL6.8 cells (Fig. 2A and Vendrell *et al.* 2005). Taken together, these observations indicate that the OTR CL6.7 and CL6.8 cellular clones obtained from MVLN cells using the same selection process (6-month 200 nM OH-Tam treatment) developed a similar resistance phenotype defined by agonist activity of OH-Tam and hypersensitivity to E₂.

OTR CL6.7 cells, but not OTR CL6.8 cells, developed cross-resistance to fulvestrant

As fulvestrant may be used as second-line therapy following Tam resistance, we investigated the proliferation response of MVLN, CL6.7, and CL6.8 cells under 100 nM fulvestrant exposure for 1, 3, 5, and 8 days (Fig. 2B). Fulvestrant treatment completely abolished the proliferation of MVLN cells detectable from the third day of treatment. Fulvestrant was also able to inhibit the proliferation of the CL6.8 cell line in a time-dependent manner, but with different kinetics. Indeed, after 3 and 5 days of treatment, CL6.8 cells were significantly less sensitive to the inhibition of cell proliferation induced by fulvestrant than MVLN cells ($P < 0.01$), but after 8 days of treatment the inhibitory action of the drug was similar in both cell lines (Fig. 2B). More surprisingly, CL6.7 cells developed total resistance to the compound, as no cytostatic activity of fulvestrant could be observed after any exposure time. The patterns of cell growth observed after exposure to both E₂ and fulvestrant for 5 days were identical to those obtained on fulvestrant alone (Fig. 2C), suggesting that in CL6.7 cells, fulvestrant is still able to prevent the E₂ stimulation of cell proliferation, but is unable to exert its cytostatic activity. As fulvestrant is known to bind to ER α and to induce the degradation of the receptor via the proteasome (Dauvois *et al.* 1992, Fan *et al.* 2003), the loss of fulvestrant-induced ER α degradation in CL6.7 cells would be an obvious explanation for the development of resistance to this molecule. As shown in Fig. 2D, fulvestrant treatment is able to induce a decrease in ER α protein expression in MVLN and CL6.8 cells, as expected, but also in the fulvestrant-resistant CL6.7 cell line. This suggests that

Table 2 Genes differentially expressed at the basal level in the CL6.7 cell line compared with the MVLN cell line, and in the VP267 compared with the VP229 cells

Gene name	Symbol	FC	
		CL6.7/MVLN	VP267/VP229
Estrogen receptors			
Estrogen receptor 1 (ER α)	<i>ESR1</i>	−1.2	−2.9
Estrogen receptor 2 (ER β)	<i>ESR2</i>	−1.6	−1.4
Cell proliferation			
Cyclin A2	<i>CCNA2*</i>	1.2	1.2
Cyclin B2	<i>CCNB2*</i>	4.2	2.6
Cyclin E2	<i>CCNE2*</i>	−2.2	−5.0
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	<i>CDKN1A*</i>	1.5	−1.5
Early growth response 2	<i>EGR2*</i>	−2.3	−7.2
Growth arrest and DNA-damage-inducible, alpha	<i>GADD45A*</i>	2.0	4.4
Insulin receptor substrate 1	<i>IRS1</i>	2.4	9.2
MAD2 mitotic arrest deficient-like 1	<i>MAD2L1*</i>	−2.5	−1.2
v-myb myeloblastosis viral oncogene homolog (avian)-like 2	<i>MYBL2*</i>	−1.2	−2.6
Nephroblastoma overexpressed gene	<i>NOV*</i>	3.7	−1.6
Transforming, acidic coiled-coil	<i>TACC1</i>	2.8	−1.8
Transformation			
Pituitary tumor-transforming 1	<i>PTTG1*</i>	1.2	2.9
Apoptosis			
BCL2-antagonist/killer 1	<i>BAK1</i>	2.0	1.7
BCL2 binding component 3	<i>BBC3*</i>	−1.1	−1.3
BCL2-interacting killer	<i>BIK*</i>	−1.3	4.6
Baculoviral IAP repeat-containing 5	<i>BIRC5*</i>	−2.8	−1.4
Death associated protein 3	<i>DAP3</i>	2.6	2.2
Transforming growth factor, β 1	<i>TGFB1</i>	−1.4	1.6
Transforming growth factor, β 2	<i>TGFB2*</i>	−2.0	−5.0
Transforming growth factor, β receptor II (70/80kDa)	<i>TGFB2</i>	3.4	−1.5
Tumor necrosis factor	<i>TNF</i>	2.4	3.5
Tumor necrosis factor receptor 1-associated death domain protein	<i>TRADD*</i>	1.9	1.1
DNA repair and DNA replication			
Breast cancer, type 2	<i>BRCA2*</i>	1.4	−1.6
Hypoxanthine guanine phosphoribosyltransferase 1	<i>HPRT1*</i>	1.3	−1.6
RAD51 homolog	<i>RAD51*</i>	−1.9	−2.2
Topoisomerase (DNA) II α 170 kDa	<i>TOP2A*</i>	1.1	2.5

The expression values presented are expressed in terms of fold change (FC) values (calculated by dividing the resistant cell line gene-expression value by the one obtained for the corresponding sensitive cell line) and represent means from at least two independent RTQ-PCR measurements. Gene-expression variation was considered significant for $FC \geq 2$ or $FC \leq -2$. Upregulations and down-regulations are indicated in bold. Asterisks represent genes on which an estrogen-like gene expression signature under OH-Tam treatment was previously demonstrated by Vendrell et al. (2005).

other unknown mechanisms are involved in the development of resistance to this SERD by CL6.7 cells.

In vivo-selected VP cell lines are another breast cancer cell model of cross-resistance to Tam and to fulvestrant

The VP229 cell line was established from a primary breast tumor removed before any pharmacological treatment, whereas VP267 was derived from the same patient after a local recurrence following Tam treatment (McCallum & Lowther 1996). These cells represent another interesting cellular model because they were selected *in vivo* under Tam treatment, but they are still not fully characterized. We explored ER α expression in the two VP cell lines, and we found a down-regulation in VP267 cells compared with VP229

cells, both at the mRNA level (−2.9-fold, Table 2) and at the protein level (Fig. 3A). *ESR2/ER β* mRNA levels were 1000-fold lower than *ESR1/ER α* mRNA levels and no difference in expression was noted between VP229 and VP267 cells (Table 2). The functionality of ER α was shown by the induction of *TFF1* (*pS2*) gene expression under E₂ stimulation in VP229 and VP267 cell lines (+49.1-fold and +13.1-fold respectively, Fig. 3B). ER-driven transcriptional activity was assessed by a gene reporter assay using an ERE-luciferase reporter gene; E₂ induced the up-regulation of luciferase activity by approximately fourfold in VP229 cells and a weak but reproducible 1.7-fold up-regulation in VP267 cells (data not shown). Finally, E₂ was capable of stimulating the proliferation of both VP229 and VP267 cells, but to a lesser extent in VP267 cells ($P<0.05$, Fig. 3C). Taken together, these data suggest that VP cell lines express a

Figure 2 Pharmacological response of MVLN, CL6.7, and CL6.8 cells under OH-Tam and fulvestrant exposure. (A) Proliferative response of MVLN (white bars), CL6.7 (black bars), and CL6.8 (grey bars) cells grown in DCC medium, then treated for 1, 3, 5, or 8 days with vehicle or 200 nM OH-Tam. (B) Proliferative response of MVLN (white bars), CL6.7 (black bars), and CL6.8 (grey bars) cells grown in DCC medium, then treated for 1, 3, 5, or 8 days with vehicle or 100 nM fulvestrant. (C) Proliferative response of MVLN (white bars), CL6.7 (black bars), and CL6.8 (grey bars) cells treated for 5 days with vehicle, 1 nM E_2 , 100 nM fulvestrant or E_2 + fulvestrant. Results are expressed as means $\pm$ s.d. from three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus the corresponding MVLN treatment according to Student's t -test. (D) Western-blot analysis of ER α expression in MVLN, CL6.8, and CL6.7 cells under 100 nM fulvestrant (F) exposure as described in the Materials and methods section using anti-ER α antibody. Expression of α -tubulin was used as invariant control.

functionally active ER α . We then explored cell response to OH-Tam and fulvestrant using a cell proliferation assay. VP229 cells, selected from a patient before any pharmacological treatment, were sensitive to the cytostatic action of both molecules (Fig. 3C). Strikingly, VP267 cells issued from a tumor recurrence under Tam

Figure 3 ER α expression and pharmacological response of VP229 and VP267 cell lines. (A) ER α protein expression was analyzed as described in the Materials and methods section using anti-ER α specific antibody. Expression of α -tubulin was used as invariant control. (B) *TFF1* (*pS2*) gene expression variations (fold change) measured by RTQ-PCR after 4 days of E_2 treatment of VP229 and VP267. (C) Proliferative response (BrdU labeling) of VP229 and VP267 cells treated for 5 days with vehicle, 1 nM E_2 (white bars), 100 nM OH-Tam (grey bars), or 1 μ M fulvestrant (black bars). * $P < 0.05$ versus the corresponding VP229 treatment according to Student's t -test. (D) Western blot analysis of the expression of ER α in VP229 and VP267 under 100 nM fulvestrant (F) exposure as described in the Materials and methods section using anti-ER α antibody. Expression of α -tubulin was used as invariant control.

treatment developed cross-resistance to both OH-Tam and fulvestrant, and these molecules stimulated VP267 cell proliferation to a degree similar to that induced by E_2 (Fig. 3C). As observed for MVLN/CL6.7 cells (Fig. 2D), fulvestrant treatment was capable of inducing a decrease in ER α protein expression in both VP229 and VP267 cell lines (Fig. 3D), suggesting again that the loss of fulvestrant-induced ER α degradation was not involved in the development of resistance to this molecule in VP267 cells.

Screening of molecular markers associated with endocrine therapy resistance in MVLN/CL6.7 and VP229/VP267 cells

As described above, while selected under Tam exposure, CL6.7, and VP267 cells also acquired cross-resistance to fulvestrant, characterized by a total abrogation of the SERD's response. Thus, the combination of CL6.7 and VP267 cells represents a good cellular model for the genomic screening of molecular markers of endocrine resistance with potential clinical relevance. To identify deregulation of gene expression associated with resistance to Tam and/or fulvestrant, we selected 26 candidate genes for RTQ-PCR analysis in MVLN/CL6.7 and VP229/VP267 cells. The genes had been identified in our previous studies as estrogen-modulated or associated with agonist activity of OH-Tam and hyper-response to E₂ in OTR breast cancer cell lines (Vendrell *et al.* 2004, 2005). The genes were selected on the basis of the biological function of the encoded proteins: cell proliferation, transformation, apoptosis, DNA repair, and DNA replication.

Analysis of the resulting RTQ-PCR data allowed the selection of 19 genes (73%) whose expression was deregulated in at least one of the two resistant cell lines (compared with the sensitive control, Table 2). We identified a variable expression (> twofold) of 14 genes (54%) between CL6.7 and MVLN cells and of 13 genes (50%) between VP267 and VP229 cells. Eight genes were similarly deregulated in the two resistant cell lines (overexpression of *CCNB2*, *GADD45A*, *IRS1*, *DAP3*, and *TNF* and underexpression of *CCNE2*, *EGR2*, and *TGFB2*) and thus might represent common molecular mechanisms developed *in vivo* or *in vitro* by breast cancer cells to escape Tam and/or fulvestrant treatment.

In our previous work (Vendrell *et al.* 2005), we demonstrated that OH-Tam stimulates the proliferation of OTR cells, and that this phenotype is associated with the appearance of an estrogen-like gene-expression signature under OH-Tam treatment (i.e. an OH-Tam agonistic action on E₂-regulated genes). This was demonstrated for 19 of the 26 genes studied in the present work (genes indicated by asterisks in Table 2). Interestingly, the expression of 13 out of these 19 genes (68%) was also deregulated at the basal level in VP267 and/or CL6.7 cells (i.e. comparison of resistant cells to their respective sensitive untreated counterparts). Taken together, these observations suggest that the occurrence of Tam-resistance has, for some genes, consequences on their expression either in the absence or under stimulation by the drug, and that the overall gene-expression variation identified in this study could represent molecular markers possibly associated with the development of endocrine resistance in breast cancer cells.

mRNA expression of the 19 candidate genes, *ESR1/ERα*, and *MKI67* in 48 breast tumor samples from patients who relapsed or not under Tam treatment

With the aim to investigate the clinical relevance of the 19 genes identified as associated with endocrine resistance in CL6.7 and/or VP267, we explored their expression levels in 48 ER+ breast tumor samples (Table 1) from postmenopausal women treated with primary surgery followed by adjuvant Tam alone. Twenty-four patients developed acquired resistance to endocrine therapy ('Relapse' group), as they relapsed under Tam treatment and developed metastases (at 1.3 to 10.0 years from surgery), while 24 patients did not ('No relapse' group). Eight out of the 19 candidate genes (42%) were significantly overexpressed in the 'Relapse' group compared with the 'No relapse' group ($P < 0.05$, Kruskal-Wallis test, Table 3): *TACCI*, *NOV*, *PTTG1*, *MAD2L1*, *BAK1*, *TGFB2*, *BIRC5*, and *CCNE2* (Table 3). The capacity of each of these eight genes to discriminate between breast tumors with and without relapse was then tested by ROC curve analysis, i.e. the overall diagnostic values of the eight molecular markers were assessed in terms of their AUC values (Table 3). *TACCI*, *NOV* and *PTTG1* emerged as the most discriminating markers of relapse on Tam (with ROC-AUC curve values of 0.899, 0.819 and 0.780 respectively). In the same set of 48 ER+ tumors, we also examined the expression of the *ESR1/ERα* gene and the *MKI67* gene (encoding the proliferation-related Ki-67 antigen) and found that their expression was not significantly different in ER+ breast tumors with or without relapse ($P > 0.05$, Kruskal-Wallis test, Table 3), suggesting that the eight candidate genes were associated with outcome independently of proliferation and ERα expression status.

Prognostic significance of *TACCI*, *NOV*, *PTTG1*, *MAD2L1*, *BAK1*, *TGFB2*, *BIRC5*, and *CCNE2*

We used univariate analysis (log-rank test) to further study the prognostic value of the eight discriminating genes and to investigate whether their expression level is correlated with prognosis and survival (Table 3). For each gene, the 48 ER+ breast tumors were divided into two equal groups of 24 tumors with 'low' (lower than the median mRNA level of the 48 breast tumor samples) and 'high' (higher than the median mRNA level of the 48 breast tumor samples) mRNA levels. Univariate analysis showed that a high expression level of *TACCI*, *NOV*, *PTTG1*, *BAK1* and *TGFB2* was correlated with significantly shorter RFS (Table 3). The *TACCI* gene emerged as the strongest prognostic marker ($P = 0.000014$, log-rank test, Table 3 and Fig. 4A). No significant prognostic value was associated

Table 3 Statistical analysis of mRNA expression of the 19 candidate genes, *ESR1/ER α* and *MKI67* in 24 ER+ breast tumors with relapse under Tam treatment relative to 24 ER+ breast tumor samples without relapse

Genes	No relapse (<i>n</i> =24)	Relapse (<i>n</i> =24)	<i>P</i> ^a	ROC-AUC ^b	Log-rank ^c
<i>TACC1</i>	1.0 (0.25–4.56) ^d	3.31 (1.11–17.75)	0.0000021	0.899	0.000014
<i>NOV</i>	1.0 (0.20–3.58)	2.71 (0.76–6.33)	0.00015	0.819	0.036
<i>PTTG1</i>	1.0 (0.26–2.01)	1.44 (0.70–13.95)	0.00087	0.780	0.014
<i>MAD2L1</i>	1.0 (0.46–2.38)	1.47 (0.21–6.53)	0.005	0.736	0.065 (NS)
<i>BAK1</i>	1.0 (0.57–4.34)	1.58 (0.76–4.68)	0.0059	0.732	0.04
<i>TGFB2</i>	1.0 (0.05–37.84)	1.88 (0.43–24.54)	0.01	0.717	0.032
<i>BIRC5</i>	1.0 (0.15–7.65)	1.99 (0.52–17.51)	0.016	0.703	0.29 (NS)
<i>CCNE2</i>	1.0 (0.42–6.22)	2.05 (0.17–6.24)	0.045	0.668	0.059 (NS)
<i>MKI67</i>	1.0 (0.09–5.52)	1.46 (0.38–8.66)	0.066 (NS)	0.655	ND ^e
<i>CCNB2</i>	1.0 (0.12–5.00)	1.40 (0.52–6.83)	0.068 (NS)	0.654	ND
<i>TOP2A</i>	1.0 (0.15–48.89)	1.22 (0.23–14.94)	0.12 (NS)	0.632	ND
<i>RAD51</i>	1.0 (0.36–4.69)	1.35 (0.34–4.35)	0.18 (NS)	0.614	ND
<i>DAP3</i>	1.0 (0.51–1.84)	1.05 (0.42–2.47)	0.38 (NS)	0.575	ND
<i>GADD45A</i>	1.0 (0.42–3.14)	1.08 (0.68–3.54)	0.39 (NS)	0.573	ND
<i>MYBL2</i>	1.0 (0.14–10.25)	1.09 (0.19–22.82)	0.51 (NS)	0.556	ND
<i>TGFBR2</i>	1.0 (0.13–4.69)	1.17 (0.36–2.22)	0.58 (NS)	0.546	ND
<i>TNF</i>	1.0 (0.04–2.96)	0.92 (0.05–4.16)	0.79 (NS)	0.523	ND
<i>BIK</i>	1.0 (0.19–2.22)	1.02 (0.23–3.64)	0.98 (NS)	0.503	ND
<i>EGR2</i>	1.0 (0.19–6.92)	0.86 (0.25–5.01)	0.86 (NS)	0.485	ND
<i>IRS1</i>	1.0 (0.11–4.18)	0.88 (0.33–4.12)	0.68 (NS)	0.465	ND
<i>ESR1/ER α</i>	1.0 (0.08–4.01)	0.55 (0.10–2.18)	0.23 (NS)	0.398	ND

NS, not significant.

^aKruskal–Wallis Test.^bROC, receiver operating characteristics; AUC, area under curve analysis.^cLog-rank test.^dMedian (range) of gene mRNA levels (normalized such that the median of the mRNA levels of the six normal breast samples would equal a value of 1).^eNot determined.

with *BIRC5*, nor with *MAD2L1* and *CCNE2* although there was a trend toward significance ($P=0.065$ and $P=0.059$ respectively, log-rank test, Table 3). Hierarchical clustering of the 48 tumor samples based on the expression of *TACC1*, *NOV* and *PTTG1* allowed the separation of the samples into two groups (groups A and B) with significantly different outcomes ($P_{\text{chi}^2}=0.0000002$; Fig. 4B): group A ($n=24$) with good clinical outcome (only 3 out of the 24 samples, i.e. 12.5%, belonged to the ‘Relapse’ group and were misclassified) and group B ($n=24$) with poor clinical outcome (21 of the 24 samples of this group, i.e. 87.5%, belonged to the ‘Relapse’ group and were thus correctly classified). The prognostic value of this three-gene expression signature was higher than the prognostic value of *TACC1* alone ($P=0.0000017$, log-rank test, Fig. 4C). Finally, using a Cox proportional hazards model, we also assessed the prognostic value for RFS of parameters that were significant or near significant ($P<0.1$) in univariate analysis, i.e. lymph-node status, macroscopic tumor size (Table 1), and *TACC1* alone (Fig. 4A) or the three-gene signature (Fig. 4C). Only the prognostic significance of *TACC1* alone and the three-gene signature persisted in Cox multivariate regression analysis of RFS ($P=0.0002$ and $P=0.0001$ respectively).

mRNA expression of *TACC1*, *NOV*, and *PTTG1* in response to OH-Tam or to fulvestrant exposure in the sensitive cell line MVLN and the cross-resistant cell line CL6.7

To further investigate the potential endocrine responsiveness of *TACC1*, *NOV*, and *PTTG1* gene expression, we analyzed by RTQ-PCR the mRNA levels of the three genes in MVLN and CL6.7 cells after 4 days of E_2 , OH-Tam or fulvestrant treatment (Fig. 5). Like *TFE1/pS2* (Fig. 1B), *TACC1*, *NOV*, and *PTTG1* responded to E_2 in both MVLN and CL6.7 cells (greater than twofold regulation; Fig. 5). The expression of *TACC1* and *NOV* was down-regulated under E_2 exposure while *PTTG1* mRNA levels were up-regulated. In MVLN cells, none of the variations produced by the pharmacological action of E_2 were observed on fulvestrant. On the contrary, fulvestrant seemed to display a ‘reversed’ pharmacology, as treatment induced reproducible down-regulation of *PTTG1* gene expression (which was up-regulated under E_2 treatment) and reproducible up-regulation of *TACC1* and *NOV* gene expression (down-regulated by E_2). The OH-Tam signature in MVLN cells was different from that of E_2 as *NOV* and *PTTG1* genes did not display any variation

Figure 4 Prognostic value of *TACC1* expression or three-gene (*TACC1*, *NOV*, and *PTTG1*) expression signature. (A) Relationship between RFS and *TACC1* expression in a series of 48 ER+ breast tumor samples. (B) Dendrogram of 48 ER+ breast tumor samples from patients who relapsed (R, $n=24$) or did not relapse (NR, $n=24$) under Tam treatment, constructed by hierarchical clustering according to the expression of *TACC1*, *NOV*, and *PTTG1*, and allowing the emergence of two groups: group A ($n=24$) and group B ($n=24$). Percentage of patients who relapsed (R) in groups A and B are indicated on the right. (C) Relationship between RFS and the three-gene expression signature (*TACC1*, *NOV*, and *PTTG1*) in a series of 48 ER+ breast tumor samples. Groups A and B correspond to the groups defined in Fig. 4B.

of expression. It was also different from that of fulvestrant as no ‘reversed’ pharmacology was observed. Finally, we detected in MVLN cells a partial agonistic action of OH-Tam on the *TACC1* gene, evidenced by a down-regulated expression on E_2 or OH-Tam treatment. Interestingly, the pharmacological responsiveness to endocrine therapy observed for the three genes in MVLN cells was totally impaired in

resistant CL6.7 cells. Indeed, in these cells, OH-Tam treatment had no impact on *TACC1* gene expression and the ‘reversed’ pharmacology of fulvestrant observed for *TACC1*, *NOV*, and *PTTG1* in MVLN cells was also totally abrogated. Taken together these data support the hypothesis that *TACC1*, *NOV*, and *PTTG1* are candidate molecular markers associated with deregulated endocrine therapy responsiveness.

Figure 5 mRNA levels of *TACCI*, *NOV*, and *PTTG1* in MVLN and CL6.7 cells under E₂, OH-Tam, and fulvestrant exposure. The gene expression variations (fold change) of (A) *TACCI*, (B) *NOV* and (C) *PTTG1* were measured by RTQ-PCR after 4 days of culture in DCC medium or under 1 nM E₂ (black bars), 200 nM OH-Tam (white bars), or 100 nM fulvestrant (grey bars). The RTQ-PCR values indicated are the means of at least three independent experiments. Gene-expression variation was considered significant for FC ≥ 2 or FC ≤ -2.

Concordance between mRNA expression levels and protein expression levels of *TACCI*, *NOV*, and *PTTG1* in cell lines and breast tumor samples

With the aim to assess the functional relevance of the biomarkers identified in this study, we explored the protein expression levels of *TACCI*, *NOV*, and *PTTG1* using specific commercially available antibodies. We aimed, first, to explore whether the *TACCI*, *NOV*, and *PTTG1* gene products are expressed at the protein level, and second, whether mRNA and protein levels are concordant. The data presented in Fig. 6A demonstrate

that all the deregulations of expression of *TACCI*, *NOV*, or *PTTG1* genes identified at the basal level in the resistant CL6.7 or VP267 cell lines, when compared with their respective sensitive cell line (Table 2), were validated by western blot analysis. In OH-Tam- or fulvestrant-treated MVLN and CL6.7 cells, most of the deregulated expression of *TACCI*, *NOV*, and *PTTG1* observed at the mRNA level (Fig. 5) and associated with aberrant endocrine therapy responsiveness was also validated at the protein level by western blot analysis (Fig. 6B). Taken together, these results demonstrate that in MVLN/CL6.7 and in VP229/VP267 cell lines, there is a very good concordance between deregulations of expression of *TACCI*, *NOV*, and *PTTG1* at the mRNA level and at the protein level.

The expression of the three candidate proteins identified above was assessed by immunohistochemistry, using antibodies directed against *TACCI*, *NOV*, or *PTTG1*, in 10 ER+ breast tumor samples. In contrast to stromal cells, specific positive staining was observed in tumor cells using the antibodies directed against *NOV* or *PTTG1* (Fig. 7). For the two proteins, the staining was localized only within the cytoplasm (Fig. 7). Unfortunately, unspecific staining was obtained with the *TACCI* antibody used in this study, thus preventing the interpretation of immunohistochemistry data. Finally, when exploring the mRNA and protein expression levels of *NOV* and *PTTG1* in each of the 10 tumor samples, we found that 80% (8/10) and 70% (7/10) respectively, of the tested samples had been correctly classified into the 'low' or the 'high' expression groups, both at the mRNA and the protein levels. Altogether, these data demonstrate, first, that *TACCI*, *NOV*, and *PTTG1* gene products are expressed and, secondly, that there is a good concordance between mRNA and protein levels for *TACCI*, *NOV*, and *PTTG1* in breast cancer cell lines, and at least for *NOV* and *PTTG1* in breast tumor samples.

Discussion

Impressive early results obtained with tamoxifen in the adjuvant setting led clinicians to use tamoxifen as neoadjuvant therapy to avoid surgery in elderly women with ER+ cancer (Horobin *et al.* 1991). However, long-term follow-up and clinical trials have demonstrated that up to 62% of cancers initially responsive to endocrine therapy subsequently escape control, with the patient requiring salvage surgery (Horobin *et al.* 1991, Fennessy *et al.* 2004). With the aim to circumvent the development of endocrine resistance and to delay chemotherapy, several clinical trials have investigated the best sequence of administration of the different molecules available for endocrine therapy as first-, second-, or third-line treatment (Howell *et al.* 2002, Osborne *et al.* 2002, Dodwell &

Figure 6 Protein detection of TACC1, NOV, and PTTG1 in MVLN/CL6.7 and VP229/VP267 cellular models of endocrine resistance. Western blot analysis of TACC1, NOV, and PTTG1 expression in (A) MVLN, CL6.7, VP229, and VP267 cells under basal condition and in (B) MVLN and CL6.7 under 200 nM OH-Tam or 100 nM fulvestrant (F) exposure. Expression of α -tubulin was used as invariant control.

Pippen 2006, Perey *et al.* 2007). Treatment with the SERD fulvestrant has proven effective upon progression on Tam but, as for Tam treatment in advanced breast cancer, resistance will inevitably occur. The molecular changes associated with fulvestrant resistance are only beginning to emerge, but previous studies have identified several proteins possibly associated with the fulvestrant resistant phenotype in cell lines: EGFR, Erbb2, Erbb3, and Erk (McClelland *et al.* 2001, Fan *et al.* 2006a, Frogne *et al.* 2008), Akt (Frogne *et al.* 2005), and NF κ B (Gu *et al.* 2002). One study has analyzed global gene expression in Tam-resistant and fulvestrant-resistant MCF-7 cells and suggested that while Tam resistance preferentially alters the expression of genes downstream of ER, fulvestrant resistance is characterized by a strong remodeling of gene expression (Fan *et al.* 2006a).

As fulvestrant is now approved for second-line treatment after failure of Tam or aromatase inhibitors, the next challenge is identifying molecular changes

associated with the development of cross-resistance to fulvestrant and thus valuable cellular models for fulvestrant cross-resistance acquisition are urgently needed. The development of such a phenotype is rarely described, as noted above. Only few studies have reported the decreased sensitivity of Tam-resistant breast cancer cells or xenografts to fulvestrant (Lykkesfeldt *et al.* 1994, Naundorf *et al.* 2000), but none, to our knowledge, has demonstrated the total loss of sensitivity to this SERD.

Our data show for the first time that both MVLN/CL6.7 and VP229/VP267 cell lines are valuable cellular models to investigate cross-resistance to endocrine therapy. Indeed, while selected in the presence of OH-Tam (or Tam) alone, CL6.7 and VP267 cells also acquired cross-resistance to fulvestrant, characterized by the total loss of fulvestrant response. Our study also demonstrated that such phenomenon can occur under *in vitro* (MVLN/CL6.7 cells) or *in vivo* (VP229/VP267

Figure 7 Analysis of NOV and PTTG1 expression in breast infiltrative carcinomas using immunohistochemistry. All images were taken at x40 magnification. (A) undetectable NOV expression level represented by the absence of cytoplasmic staining, (B) low NOV expression level represented by a focal 1+ cytoplasmic staining, (C) high NOV expression level represented by a diffuse 3+ cytoplasmic staining, (D) undetectable PTTG1 expression level represented by the absence of cytoplasmic staining, (E) low PTTG1 expression level represented by a 1+ cytoplasmic staining, and (F) high PTTG1 expression level represented by a 2+ cytoplasmic staining.

cells) selection. We also showed that acquisition of such phenotype was not due to any loss in ER α functionality or any loss in fulvestrant binding to ER α . Finally, our study also suggests that under the same selection process (OH-Tam selection), the resulting resistant cellular clones can display different endocrine therapy resistance phenotypes, as demonstrated for the two cellular clones CL6.8 and CL6.7 which, while issued from the same selection process (Vendrell *et al.* 2005), developed different modes of acquisition of resistance to endocrine therapy: OH-Tam resistance only (CL6.8 cells) or cross-resistance to OH-Tam and fulvestrant (CL6.7 cells). This probably reflects the heterogeneity of breast cancer cells in tumors having acquired resistance to first-line Tam endocrine therapy and thus suggests that, in these tumors, cells that will be refractory to fulvestrant second-line therapy are already present after the first selection. This has important clinical consequences because, while the difference between the mechanisms of action of Tam and fulvestrant (Wakeling *et al.* 1991, Tzukerman *et al.* 1994, McDonnell *et al.* 1995, Dowsett *et al.* 2005) supports the use of fulvestrant as second-line treatment in Tam-resistant patients (Howell *et al.* 2002, Osborne *et al.* 2002), cross-resistance to fulvestrant may also occur in Tam-resistant patients. Thus, there is a pressing need for identifying general biomarkers of endocrine therapy resistance to help in the clinical management of such patients.

As MVLN/CL6.7 cells and VP229/VP267 cell lines are original and valuable models of cross-resistance to Tam and fulvestrant, identifying gene expression

deregulations in these cell lines would represent a significant step towards the identification of new biomarkers. Of the 26 genes screened by RTQ-PCR, 19 displayed a deregulation of expression in at least one of the two cross-resistant cell lines tested, and two, *TGFB2* and *TGFB2R*, have also been recently identified in *in vitro* models of Tam- or fulvestrant-resistance (Fan *et al.* 2006a). Several genes (*CCNB2*, *CCNE2*, *EGR2*, *GADD45A*, *IRS1*, *DAP3*, *TGFB2*, and *TNF*) which were found similarly deregulated in both resistant cell lines, might represent common molecular mechanisms developed *in vivo* and *in vitro* by breast cancer cells to escape Tam and/or fulvestrant treatment. Indeed, all of these genes belong to two functional clusters previously described as associated with poor endocrine response (Paik *et al.* 2004, Jansen *et al.* 2005, Chanrion *et al.* 2008): *CCNB2*, *CCNE2*, *EGR2*, *GADD45A*, and *IRS1* belong to the cell proliferation functional cluster and *DAP3*, *TGFB2*, and *TNF* to the apoptosis functional cluster. A supporting observation is that *CCNB2* has previously been described as part of a 36-gene signature predictive of Tam recurrence (Chanrion *et al.* 2008).

In a well-defined cohort of 48 relapsing or non-relapsing ER+ postmenopausal breast cancer patients treated with primary surgery followed by adjuvant Tam alone, we found that 8 out of the 19 genes selected on cross-resistant breast cancer cells (*TACCl*, *NOV*, *PTTG1*, *MAD2L1*, *BAK1*, *TGFB2*, *BIRC5*, and *CCNE2*) were significantly overexpressed in the group of patients who relapsed under Tam treatment ($P < 0.05$, Kruskal-Wallis test). Interestingly, 3 out of these 8 are mitotic checkpoint genes (*TACCl*, *PTTG1*, and *MAD2L1*).

BIRC5 also belongs to the 21-gene Tam recurrence signature described by Paik and colleagues (Paik *et al.* 2004), and *CCNE2* to the 70-gene prognostic signature identified by van 't Veer *et al.* (2002).

In the present work, multivariate analysis demonstrated that *TACC1* alone and the three-gene expression signature *TACC1*, *NOV*, and *PTTG1* were independent prognostic markers (log-rank test, Cox multivariate analysis). The transforming acidic coiled coil 1 (*TACC1*), which emerged as the strongest prognostic marker, is a putative oncogene located within a breast cancer amplicon found on human chromosome 8p11 (Still *et al.* 1999) which we have previously identified as a new estrogen-regulated gene (Vendrell *et al.* 2004). The *TACC1* protein is involved in mitotic checkpoint regulation through stabilization of microtubules in the growing spindle, and interacts with the Aurora A/STK6 kinase (Conte *et al.* 2003). *TACC1* can transform fibroblasts (Still *et al.* 1999), and recent work has shown that it promotes transformation and mammary tumorigenesis through positive regulation with the Ras and PI3K signaling pathways (Cully *et al.* 2005), both also involved in the development of Tam resistance (for review, Moy & Goss 2006). More interestingly, high levels of amplification of the 8p11 region (encoding the *TACC1* gene) have been significantly associated with reduced survival duration or distant recurrence in breast cancer (Chin *et al.* 2006). *NOV* (neuroblastoma overexpressed gene), which encodes a negative regulator of cell proliferation, can be involved in tumorigenesis (Scholz *et al.* 1996). In a previous work, we have identified *NOV* as a new estrogen-regulated gene (Vendrell *et al.* 2004) and also a gene on which Tam exerts an estrogen-like activity in Tam-resistant breast cancer cells (Vendrell *et al.* 2005). Several studies have reported an association between low expression levels of *NOV* and progression of adrenocortical tumors (Martinerie *et al.* 2001), renal cell carcinoma (Niu *et al.* 2005), and poor breast cancer prognosis (Jiang *et al.* 2004). In this study, we show that high expression levels of *NOV* are associated with endocrine therapy cross-resistance in CL6.7 cells (Table 2) and endocrine therapy resistance in breast tumor samples (Table 3). Further investigations are needed to explain this specific point. Finally, *PTTG1* (pituitary tumor-transforming gene 1, also called Securin) encodes a regulatory protein that plays a central role in mitotic checkpoint control, chromosome stability, the p53 pathway and DNA repair. *PTTG1* negatively regulates the transcriptional activity and related apoptotic activity of p53, which explains the strong transforming capability of this protein when it is over-expressed (Bernal *et al.* 2002). In a previous work, we have demonstrated that *PTTG1* gene expression is associated with hypersensitivity to E₂ and agonist-like activity of Tam in Tam-resistant breast cancer cells (Vendrell *et al.* 2005). Recently, two single-nucleotide polymorphisms in the *PTTG1* gene have been associated with increased risk of

breast cancer (Lo *et al.* 2007). Furthermore, increased expression of *PTTG1* has been correlated with poor prognosis in glioma patients and in hepatocellular carcinoma (Fujii *et al.* 2006, Genkai *et al.* 2006).

Taken together, these data provide evidence that *TACC1*, *NOV*, and *PTTG1* genes are new biomarkers associated with endocrine therapy resistance in breast cancer. None of these three genes belongs to the 21-gene Tam recurrence signature described by Paik and colleagues (Paik *et al.* 2004), to the gene expression signature that predicts the clinical outcome of breast cancer patients treated with tamoxifen (Jansen *et al.* 2005), to the two-gene signature described by Ma and colleagues (Ma *et al.* 2004), or to the predictive gene expression signature of Tam recurrence defined more recently by Chanrion and colleagues (Chanrion *et al.* 2008). Fan *et al.* (2006b) have compared the predictions derived from five gene expression profiling studies and suggested that, even though there is little gene overlap between the different studies using different algorithms, the outcome predictions are similar and probably track the same phenotype. This suggests that although there is no overlap between the present study and other published studies, the three genes selected here may still be compatible. The supporting argument is that these three genes belong to a functional cluster (proliferation/transformation) present in previously published classifiers (van 't Veer *et al.* 2002, Paik *et al.* 2004, Jansen *et al.* 2005, Chanrion *et al.* 2008).

In conclusion, the identification of more specific new markers to better predict breast cancer outcome and response to endocrine therapy appears fundamental and clinically useful, as it would allow personalizing the patient's treatment. Genomic approaches based on large-scale experiments have been successfully used in breast cancer tumors to identify prognostic markers such as aberrant gene expression signatures or genomic alterations associated with Tam failure (Ma *et al.* 2004, Paik *et al.* 2004, Jansen *et al.* 2005, Han *et al.* 2006, Chanrion *et al.* 2008). An alternative approach is based on the exploration of a limited number of candidate genes by RTQ-PCR. This technique, which focuses on a small number of genes, has already proved useful for the identification of candidate gene expression signatures with prognostic significance. Using RTQ-PCR, Paik and colleagues have been able to identify a 21-gene signature that could predict recurrence of tamoxifen-treated breast cancer (Paik *et al.* 2004). In this study, we selected candidate genes identified by previous cDNA array experiments and we explored by RTQ-PCR their expression in cellular models of endocrine therapy cross-resistance and in breast tumor samples. We were able to identify *TACC1*, *NOV*, and *PTTG1* as prognostic markers associated with endocrine therapy resistance in breast tumor samples. Future work on a larger cohort is needed to validate their prognostic value but also their

predictive value for fulvestrant response in patients developing resistance to first-line Tam treatment. Moreover, as aromatase inhibitors may replace Tam for first-line therapy in postmenopausal women, investigating the expression of these new biomarkers in aromatase inhibitor-resistant breast tumor samples would be very informative, as it would allow to delineate the overlap between expression signatures of resistance to ER-ligands (Tam and fulvestrant) and to aromatase inhibitors. Encouraging results have been obtained, since we have shown a good correlation between the mRNA and protein levels of TACC1, NOV, and PTTG1 in cell lines and/or breast tumor samples, suggesting that they could be considered as candidate protein markers for immunohistochemical analysis. The present work is thus a first step towards the identification of new biomarkers associated with endocrine resistance that could be used in the clinical management of ER+ breast cancer patients.

Declaration of interest

All co-authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research report.

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