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Identification of *CGA* as a Novel Estrogen Receptor-responsive Gene in Breast Cancer: An Outstanding Candidate Marker to Predict the Response to Endocrine Therapy[1]

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ABSTRACT

The estrogen receptor (ER) status of breast tumors is used to identify patients who may respond to endocrine agents such as tamoxifen. However, ER status alone is not perfectly predictive, and there is a pressing need for more reliable markers of endocrine responsiveness.

Here, we identified the well-known *CGA* gene (coding for the α subunit of glycoprotein hormones) as a new ERα-responsive gene in human breast cancer cells. We used a real-time quantitative reverse transcription-PCR assay to quantify *CGA* mRNA copy numbers in a large series of breast tumors. *CGA* overexpression (>10 SD above the mean for normal breast tissues) was observed in 44 of 131 (33.6%) breast tumor RNAs, ranging from 20 to 16,500 times the level in normal breast tissues; the highest levels of *CGA* gene expression were close to those observed in placenta.

Significant links were observed between *CGA* gene overexpression and Scarff-Bloom-Richardson histopathological grade I+II ($P = 0.015$), and progesterone ($P = 0.0009$) and estrogen ($P < 10^{-7}$) receptor positivity, which suggested that *CGA* is a marker of low tumor aggressiveness. We observed *CGA* mRNA overexpression in 44 of 90 (48.9%) ERα-positive tumors and in none of the 41 ERα-negative tumors. Immunohistochemical studies demonstrated that human chorionic gonadotropin α protein was strictly limited to ERα-positive tumor cells. Overexpression of the *CGA* gene was not accompanied by overexpression of the *CGB* gene.

Our results also suggest that *CGA* could be a more reliable marker than *PS2* and *PR* for ERα functionality and, thus, for endocrine responsiveness. Moreover, the *CGA* marker has the added value of dichotomizing ERα-positive patients into two subgroups of similar size. Specific antibodies directed to secreted human chorionic gonadotropin α protein are commercially available, thus facilitating the future application of this marker to the clinical management of breast cancer.

INTRODUCTION

Breast cancer growth is regulated by estrogen, whose effect is mediated by binding to the ER.[3] The presence of ER in breast tumors has thus been used to identify those patients who may respond to endocrine agents such as tamoxifen. However, one-half of the patients with ER-positive tumors fail to respond favorably to antiestrogen treatment (1). Several mechanisms have been put forward to explain the lack of response in ER-positive patients; one postulates a mutated, nonfunctional ER that is unable to mediate the inhibitory cellular actions of endocrine treatments (2). It has been postulated that coassessment of ER- and estrogen-inducible genes or protein products (as markers of functional ER-mediated cellular growth mechanisms) might give better predictive results. Measurement of tumor PR and PS2 contents have been shown to partly improve patient selection (3). However, expression of PR and PS2 correlate strongly with ER expression, meaning that the PS2 and PR markers provide little further information on hormone dependence relative to ER. There is a pressing need for more reliable markers of endocrine responsiveness, but few ER-responsive genes have been identified.

We fortuitously identified the well-known *CGA* gene (coding for the α subunit of glycoprotein hormones) as a new ERα-responsive gene in human breast cancer cells. Indeed, the initial purpose of this study was to determine whether the emergence of "trophoblastic" *CGB* genes, which we have previously shown to be associated with malignant breast transformation (4), was accompanied by cooverexpression of the *CGA* gene and the production of ectopic hCG heterodimeric hormone in breast tumor cells. We thus quantified *CGA* and *CGB* gene expression in the large series of unilateral invasive primary breast tumor RNAs by means of real-time quantitative RT-PCR assays based on TaqMan methodology. We unexpectedly found marked differences in the total amount of *CGA* mRNA molecules that were clearly independent of *CGB* expression status but fully associated with the ERα-positive phenotype in our breast tumor series. Indeed, *CGA* gene overexpression was observed in one-half of the ERα-positive tumors and in none of the ERα-negative tumors. Immunohistochemical studies confirmed the hCGα protein overexpression and showed that it was strictly limited to ERα-positive tumor cells.

To further examine the unexpected link between *CGA* gene overexpression and ERα positivity, we analyzed the expression of the estrogen-responsive genes *PR* and *PS2*. Our results suggest that *CGA* could be a more reliable marker than *PR* and *PS2* for ERα functionality and, thus, for endocrine responsiveness.

MATERIALS AND METHODS

Patients and Samples

We analyzed tissue from primary breast tumors excised from 131 women treated at the Center René Huguenin from 1977 to 1989. 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 more than 60%. Immediately after surgery, the tumor samples were stored in liquid nitrogen until RNA extraction.

Immunohistochemical study was performed on fixed, paraffin-embedded tissue sections from 20 of the 131 breast tumor specimen.

The patients (mean age, 58.2 years; range, 34–91) met the following criteria: primary unilateral nonmetastatic breast carcinoma on which complete clinical, histological, and biological data were available; and no radiotherapy or chemotherapy before surgery. The main prognostic factors are presented in Table 1. The median follow-up was 8.1 years (range, 1.0–15.9). Forty-seven

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[3] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; hCG, human chorionic gonadotropin; RT-PCR, reverse transcription-PCR; EIA, enzyme immunoassay; SBR, Scarff-Bloom-Richardson; ERE, estrogen-response element; CRE, cAMP response element; CREB, CRE binding protein; CBP, CREB binding protein.

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Table 1 *Characteristics of the 131 patients and relation to disease-free survival*

	No. of patients (%)	Disease-free survival: No. of events[a]	P^b
Age			NS[c]
≤50	39 (29.8)	12	
>50	92 (70.2)	35	
Menopausal status			NS
Premenopausal	45 (34.3)	16	
Postmenopausal	86 (65.7)	31	
Histological grade[d]			NS
I+II	76 (62.3)	30	
III	46 (37.7)	16	
Lymph node status			0.026
Node-negative	49 (37.4)	10	
Node-positive	82 (62.6)	37	
ER status			NS
+ (≥10 fm/mg)	90 (68.7)	36	
− (<10 fm/mg)	41 (31.3)	11	
PR status			NS
+ (≥10 fm/mg)	78 (59.5)	28	
− (<10 fm/mg)	53 (40.5)	19	
Macroscopic tumor size[e]			NS
≤30 mm	90 (72.6)	32	
>30 mm	34 (27.4)	13	

[a] First relapses (local and/or regional recurrences, and/or metastases).
[b] Log-rank test.
[c] NS, not significant.
[d] SBR classification; information available for 122 patients.
[e] Information available for 124 patients.

patients relapsed (the distribution of first relapse events was as follows: 13 local and/or regional recurrences, 30 metastases, and 4 both).

Specimens of adjacent normal breast tissue from nine of the breast cancer patients, and normal breast tissue from six women undergoing cosmetic breast surgery, were used as sources of normal RNA.

Evaluation of "Classical" Prognostic Factors

The histological type and steroid-hormone receptor status of each tumor, and the number of positive axillary nodes, were established at the time of surgery. The malignancy of infiltrating carcinomas was scored according to Bloom and Richardson's histoprognostic system (5). ER and PR status was assayed by biochemical method (EIA) as described by the European Organization for Research and Treatment of Cancer (6), with a detection limit of 10 fmol/mg cytosolic protein.

Real-Time RT-PCR

Theoretical Basis. Quantitative values are obtained from the threshold cycle number at which the increase in the signal associated with exponential growth of PCR products begins to be detected using PE Biosystems analysis software, according to the manufacturer's manuals.

The precise amount of total RNA added to each reaction mix (based on absorbance) and its quality (*i.e.,* lack of extensive degradation) are both difficult to assess. We, therefore, also quantified transcripts of the *RPLP0* gene (also known as *36B4*) encoding human acidic ribosomal phosphoprotein P0 as the endogenous RNA control, and each sample was normalized on the basis of its *RPLP0* content.

The relative target gene expression level was also normalized to a calibrator, or 1× sample, consisting of a normal breast tissue sample (quantitation of *CGA*, *CGB*, and *RPLP0* genes) or of the breast tumor tissue sample that contained the smallest amount of target gene mRNA (quantitation of other target genes).

Final results, expressed as *N*-fold differences in target gene expression relative to the *RPLP0* gene and the calibrator, termed N*target*, were determined as follows:

$$\mathrm{N}target = 2^{(\Delta Ct_{\mathrm{sample}} - \Delta Ct_{\mathrm{calibrator}})} \quad \text{(A)}$$

where ΔCt values of the sample and calibrator are determined by subtracting the average Ct value of the target gene from the average Ct value of the *RPLP0* gene.

Primers, Probes, and PCR Consumables. Primers and probes for the *RPLP0* and target genes were chosen with the assistance of the computer programs Oligo 4.0 (National Biosciences, Plymouth, MN) and Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA). We conducted BLASTN searches against dbEST, htgs, and nr (the nonredundant set of the GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan database sequences) to confirm the total gene specificity of the nucleotide sequences chosen for the primers and probes, and the absence of DNA polymorphisms. The nucleotide sequences of the oligonucleotide hybridization probes and primers are shown in Table 2. To avoid amplification of contaminating genomic DNA, one of the two primers was placed in a different exon. For example, the upper primer and the probe of *CGA* were placed in exon 3, and the lower primer was placed at the junction between exons 3 and 4.

RNA Extraction. Total RNA was extracted from breast specimens by using the acid-phenol guanidium method. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and 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 buffer [500 mM each dNTP, 3 mM MgCl2, 75 mM KCl, and 50 mM Tris-HCl (pH 8.3)], 10 units of RNasinTM RNase inhibitor (Promega, Madison, WI), 10 mM DTT, 50 units of Superscript II RNase H-reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 1.5 mM random hexamers (Pharmacia, Uppsala, Sweden), and 1 μg of total RNA. The 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 an ABI Prism 7700 Sequence Detection system (Perkin-Elmer Applied Biosystems). PCR was performed using either the TaqMan PCR Core Reagents kit or the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C

Table 2 *Oligonucleotide primer and probe sequences used*

Genes	Oligonucleotide	Sequence	PCR product size (pb)
RPLP0	Upper primer	5′-GGC GAC CTG GAA GTC CAA CT-3′	149
	Lower primer	5′-CCA TCA GCA CCA CAG CCT TC-3′	
	Probe	5′-ATC TGC TGC ATC TGC TTG GAG CCC A-3′	
CGA	Upper primer	5′-TCC CAC TCC ACT AAG GTC CAA-3′	106
	Lower primer	5′-CCC CAT TAC TGT GAC CCT GTT-3′	
	Probe	5′-CAC AGC AAG TGG ACT CTG AGG TGA CG-3′	
CGB	Upper primer	5′-GCT ACT GCC CCA CCA TGA CC-3′	94
	Lower primer	5′-ATG GAC TCG AAG CGC ACA TC-3′	
	Probe	5′-CCT GCC TCA GGT GGT GTG CAA CTA CC-3′	
ERα[a]	Upper primer	5′-CCA CCA ACC AGT GCA CCA TT-3′	108
	Lower primer	5′-GGT CTT TTC GTA TCC CAC CTT TC-3′	
PR[a]	Upper primer	5′-CGC GCT CTA CCC TGC ACT C-3′	121
	Lower primer	5′-TGA ATC CGG CCT CAG GTA GTT-3′	
PS2[a]	Upper primer	5′-CAT CGA CGT CCC TCC AGA AGA G-3′	105
	Lower primer	5′-CTC TGG GAC TAA TCA CCG TGC TG-3′	
ERβ[a]	Upper primer	5′-AGA GTC CCT GGT GTG AAG CAA G-3′	143
	Lower primer	5′-GAC AGC GCA GAA GTG AGC ATC-3′	

[a] The genes *ERα*, *PR*, *PS2*, and *ERβ* were analyzed by using the fluorescent SYBR green methodology (without use of a TaqMan fluorogenic probe).

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for 10 min and 50 cycles at 95°C for 15 s and 65°C for 1 min. Experiments were performed with duplicates for each data point.

Immunohistochemical Studies

Indirect immunoperoxidase staining of fixed tissues was performed using monoclonal antibody HT13 directed against the α subunit of glycoprotein hormones [a kind gift from Dr Jean-Michel Bidart (Service de Biologie Oncologique, Institut Gustave Roussy, 94805 Villejuif Cedex, France); Ref. 7] and monoclonal antibody ID5 raised against human ER (Dako SA, Trappes, France).

The immunohistochemical procedure was performed on paraffin-embedded tissue sections. A microwave antigen-retrieval technique was used in all of the cases. Sections were mounted on precoated slides (triethylenethiophosphoramide-coated slides) and allowed to dry at 50°C overnight. The sections were then dewaxed in xylene and hydrated through graded concentrations of alcohol. Endogenous activity was blocked with 1% hydrogen peroxide for 15 min. Sections were then immersed in a thermoresistant plastic box containing 10 ml of citrate buffer (pH 6.0) and processed in the microwave oven four times for 5 min each at 750 W. Sections were then allowed to cool at room temperature for 30 min before rinsing in TRIS-buffered saline. The blocking reagent was topped off, and the primary antibodies were left for 1 h. A standard avidin-biotin-peroxidase complex was used to reveal the antibody-antigen reaction.

Localization and intensity of staining were assessed by two pathologists, blinded to the real-time RT-PCR results.

Statistical Analysis

Relapse-free survival was determined as the interval between diagnosis and detection of the first relapse (local and/or regional recurrences, and/or metastases).

Clinical, histological, and biological parameters were compared using the χ^2 test. Differences between the two populations were judged significant at confidence levels greater than 95% ($P < 0.05$). Survival distributions were estimated by the Kaplan-Meier method (8), and the significance of differences between survival rates was determined using the log-rank test.

Table 3 *Relationship between mRNA CGA status (normal/overexpression) and the standard clinical, pathological, and biological factors*

		Normal *CGA* mRNA	Overexpressed *CGA* mRNA	
	Total population (%)	No. of patients (%)	No. of patients (%)	P^a
Total	131 (100.0)	87 (66.4)	44 (33.6)	
Age				NS[b]
≤50	39 (29.8)	29 (33.3)	10 (20.7)	
>50	92 (70.2)	58 (66.7)	34 (77.3)	
Menopausal status				NS
Premenopausal	45 (34.3)	33 (37.9)	12 (27.3)	
Postmenopausal	86 (65.7)	54 (62.1)	32 (72.7)	
Histological grade[c]				0.015
I+II	76 (62.3)	43 (54.4)	33 (76.7)	
III	46 (37.7)	36 (45.6)	10 (23.3)	
Lymph node status				NS
Node-negative	49 (37.4)	33 (37.9)	16 (36.4)	
Node-positive	82 (62.6)	54 (62.1)	28 (63.6)	
ER status				$<10^{-7}$
+ (≥10 fm/mg)	90 (68.7)	46 (52.9)	44 (100.0)	
− (<10 fm/mg)	41 (31.3)	41 (47.1)	0	
PR status				0.0009
+ (≥10 fm/mg)	78 (59.5)	43 (49.4)	35 (79.5)	
− (<10 fm/mg)	53 (40.5)	44 (50.6)	9 (20.5)	
Macroscopic tumor size[d]				NS
≤30 mm	90 (72.6)	62 (73.8)	28 (70.0)	
>30 mm	34 (27.4)	22 (26.2)	12 (30.0)	
Relapses				NS
+	47 (35.9)	32 (36.8)	15 (34.1)	
−	84 (64.1)	55 (63.2)	29 (65.9)	
RNA *CGB* status				NS
N*CGB* (0.3–2.9)	33 (25.2)	25 (28.7)	8 (18.2)	
N*CGB* (3.0–5.9)	31 (23.7)	20 (23.0)	11 (25.0)	
N*CGB* (6.0–9.9)	35 (26.7)	23 (26.4)	12 (27.3)	
N*CGB* (10.0–192)	32 (24.4)	19 (21.9)	13 (29.5)	
RNA *CCND1* status[e]				0.00003
Overexpressed	43 (32.8)	18 (20.7)	25 (56.8)	
Normal	88 (67.2)	69 (79.3)	19 (43.2)	
RNA *MYC* status[f]				NS
Overexpressed	28 (21.4)	17 (19.5)	11 (25.0)	
Normal	103 (78.6)	70 (80.5)	33 (75.0)	
RNA *ERBB2* status[g]				NS
Overexpressed	22 (16.8)	16 (18.3)	6 (13.6)	
Normal	109 (83.2)	71 (81.7)	38 (86.4)	

[a] χ^2 test.
[b] NS, not significant.
[c] SBR classification; information available for 122 patients.
[d] Information available for 124 patients.
[e] I. Bièche, M. Olivi, C. Noguès, M. Vidaud, and R. Lidereau. Prognostic value of *CCND1* gene status in sporadic breast tumors, as determined by real-time quantitative PCR assays, manuscript in preparation.
[f] I. Bièche *et al.,* (9).
[g] I. Bièche *et al.* (10).

RESULTS

***CGA* mRNA Level in Normal Breast Tissues.** To determine the cutoff point for altered *CGA* gene expression at the RNA level in breast cancer tissue, the N*CGA* value, calculated as described in "Materials and Methods," was determined for 15 normal breast tissue RNAs. As this value consistently fell between 0.4 and 6.6 (mean, 1.83 ± 1.78 SD), values of 20 (mean + 10 SD) or more were considered to represent *CGA* gene overexpression in tumor RNA samples.

***CGA* mRNA Level in Tumor Breast Tissues.** Among the 131 breast tumor RNA samples tested, 44 (33.6%) showed *CGA* gene overexpression. Very large differences in the amount of *CGA* mRNA were observed (N*CGA*, from 20.6 to 16500): 10 tumors had an expression level of 20–40 times; 11 tumors 41–100 times; 13 tumors 101–1000 times; and 10 tumors >1000 times that of normal breast tissue RNA. The highest levels of *CGA* gene expression in breast tumors were close to those observed in placenta (data not shown). *CGA* gene expression was also investigated in nine patients from whom both primary breast tumors and matched normal breast tissue were available: four tumors showed clearly higher *CGA* expression in the tumor (N*CGA*: 2260.1, 70.4, 66.9, and 36.9, respectively) than in the normal tissue (N*CGA*: 0.9, 3.4, 2.2, and 1.3, respectively).

Correlation between *CGA* mRNA Levels and Clinical and Pathological Parameters. We then sought links between quantitative *CGA* mRNA status and standard clinical, pathological, and biological factors in breast cancer (Table 3).

Statistically significant links were found between *CGA* gene overexpression and SBR histopathological grade I+II ($P = 0.015$), and positive progesterone ($P = 0.0009$) and estrogen ($P < 10^{-7}$) receptor status, as determined by biochemical method (EIA). Importantly, we observed an absolute link between *CGA* gene overexpression and ER status, 44 of 90 (48.9%) ER-positive tumors and none of the 41 ER-negative tumors showed *CGA* gene overexpression.

Patients with tumors overexpressing *CGA* did not relapse more (or less) frequently (Table 3) and did not have significantly shorter (or longer) disease-free survival after surgery compared with patients whose tumors normally expressed *CGA* (log-rank test, not shown).

Localization of hCGα Subunit in Tumor Cell Cytoplasm. Of the 20 tumors studied by immunohistochemistry, we detected specific immunoreactivity in the 10 tumors that overexpressed *CGA* mRNA and in none of the tumors that showed no *CGA* overexpression. We thus observed a perfect match between *CGA* mRNA overexpression and immunohistochemical positivity (Fig. 1). hCGα immunoreactivity was exclusively found inside ER-positive tumor cells; infiltrating lymphocytes and normal glandular cells in the tumor were negative. Tumor cell staining was found exclusively in the cytoplasm, and the staining pattern consisted mainly of isolated positive cells (Fig. 1). The positive cells were chromogranin A-negative (data not shown).

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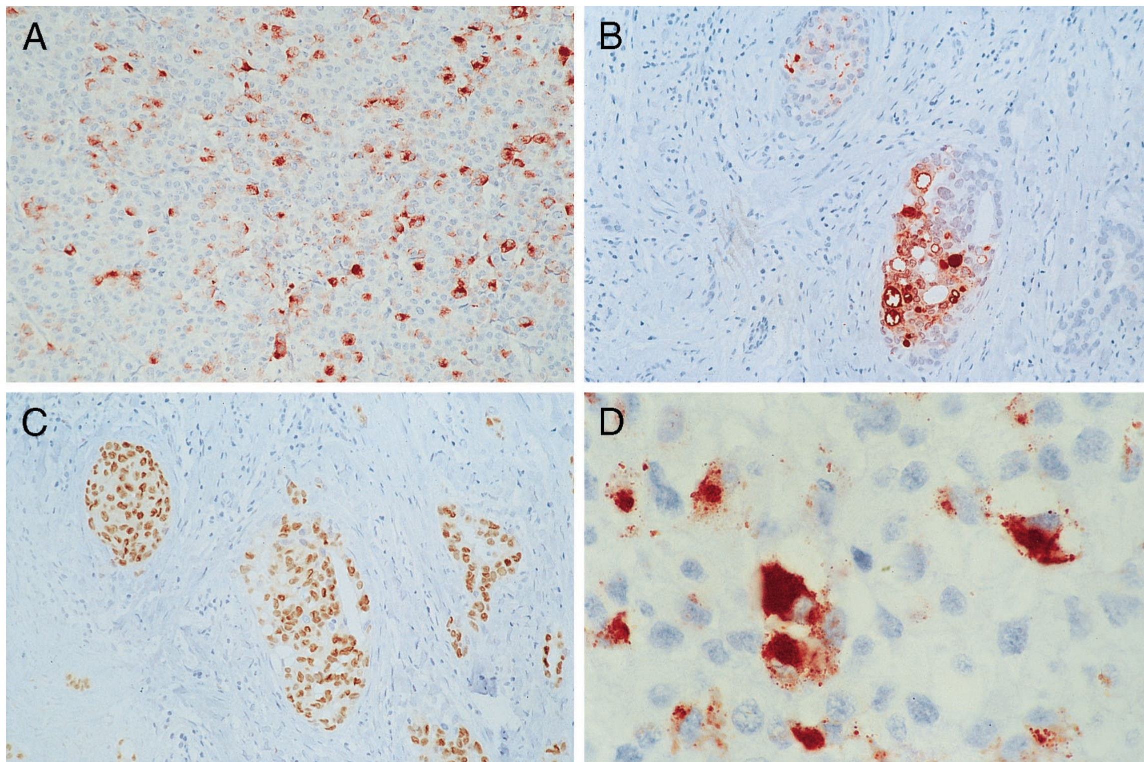

Fig. 1. Immunohistochemistry staining of hCGα (*A*, *B*, and *D*) and ERα (C) proteins in breast cancer tissues. *A* and *B*, intense expression of hCGα protein was found mainly in isolated breast tumor cells (*A*) or in cribriform areas (*B*). ×20. *B* and *C*, serial sections show hCGα immunoreactivity (*B*) exclusively found inside ERα-positive tumor cells (*C*). Note a negative staining of hCGα protein in some ERα-positive tumor cells. ×20. *D*, hCGα immunoreactivity was localized in the cytoplasm of the cancer cells; ×100.

Relationship between *CGA* mRNA Levels and *CGB* mRNA Levels. Major differences in the amount of *CGB* mRNA molecules were observed (range, 0.3–192). However, the highest levels of *CGB* gene expression in breast tumors were $>10^3$-fold lower than those observed in placenta (data not shown). To study the relationship between *CGA* mRNA levels and *CGB* mRNA levels, we subdivided tumors into those with very low (N*CGB*, 0.3–2.9), low (3–5.9), intermediate (6–9.9) and high (10–192) *CGB* mRNA levels. No correlation was observed between *CGA* mRNA and *CGB* mRNA levels (Table 3). Neither were links observed between *CGB* gene overexpression and PR and ER status (data not shown).

Relationship between *CGA* mRNA Levels and *CCND1*, *MYC*, and *ERBB2* Expression Status. The 131 tumors studied for *CGA* expression had previously been tested for mRNA expression of the *CCND1*, *MYC*, and *ERBB2* genes (9, 10).[4] We found a significant link between *CGA* overexpression and *CCND1* overexpression ($P = 0.00003$) but no link between *CGA* overexpression and altered mRNA expression of the other two genes (Table 3).

Relationship between *CGA* mRNA Levels and *PS2* and *ERβ* Expression Status. To study the link between *CGA* gene overexpression and ER positivity, we also quantified, by means of a real-time quantitative RT-PCR assay, the expression of the ERα-responsive gene *PS2*, as well as the ER β (*ERβ*) gene. We also analyzed mRNA levels of the *PR* and *ERα* genes to confirm PR and ER protein status determined by a biochemical (EIA) method, to obtain quantitative *PR* and *ERα* values and to avoid a possible discrepancy due to tumor heterogeneity.

As regards the *ERα*, *PS2*, *ERβ*, and *PR* markers, patients were subdivided into three equal groups of tumors with low ($n = 43$), intermediate ($n = 44$), and high ($n = 44$) mRNA levels. A total correlation was observed between *ERα* gene status determined with biochemical and real-time RT-PCR. Indeed, all of the 41 ER-negative tumors in the biochemical (EIA) method had low *ERα* mRNA levels. Then, breast tumors were subdivided into *ERα*-negative tumors (low *ERα* mRNA; $n = 43$) and *ERα*-positive tumors (intermediate/high *ERα* mRNA; $n = 88$).

The results (summarized in Tables 4 and 5) showed a stronger association between ERα gene status and *CGA* gene status (total link) than between *ERα* gene status and *PS2*, *PR* or *ERβ* gene status (subtotal links). Indeed, several *ERα*-negative (low *ERα* mRNA expressed) tumors overexpressed *PS2* and/or *PR*, whereas none of the "*ERα*-negative" tumors overexpressed the *CGA* gene. Interestingly, we observed a negative correlation between *ERα* and *ERβ* mRNA expression levels ($P = 0.0013$).

Finally, in the *ERα*-positive subgroup ($n = 88$), we found a significant link between *CGA* overexpression and *CCND1* overexpression ($P = 0.0024$), but no link between *CGA* mRNA expression status and *PS2*, *PR,* or *ERβ* mRNA expression levels (Table 6).

DISCUSSION

In this report, we have developed a real-time quantitative RT-PCR assay to quantify *CGA* mRNA copy numbers in homogeneous total RNA solutions prepared from tumor samples. This recently developed approach to nucleic acid quantification in homogeneous solutions may become a reference, given its performance, accuracy, sensitivity, and high throughput capacity, and also the fact that it eliminates the need for tedious post-PCR processing (11). Above all, this method is suited to the development of new target gene assays with a high level of interlaboratory standardization and yields statistical confidence values. A major advantage of this method is that the system has a large

[4] I. Bièche, M. Olivi, C. Noguès, M. Vidaud, and R. Lidereau. Prognostic value of *CCND1* gene status in sporadic breast tumors, as determined by real-time quantitative PCR assays, manuscript in preparation.

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Table 4 *Relationship between mRNA ERα status and other molecular marker status*

	Total population	*ERα*-negative tumors Low *ERα* mRNA expressed tumors (N*ERα*: 1–40)[a]	*ERα*-moderately positive tumors Intermediate *ERα* mRNA expressed tumors (N*ERα*: 41–300)	*ERα*-highly positive tumors High *ERα* mRNA expressed tumors (N*ERα*: 301–50,000)	P^b
Total	131 (100.0)[c]	43 (32.8)	44 (33.6)	44 (33.6)	
CGA gene mRNA					$<10^{-7}$
Normal	87 (66.4)	43 (100.0)	22 (50.0)	22 (50.0)	
Overexpressed	44 (33.6)	0	22 (50.0)	22 (50.0)	
PS2 gene mRNA					$<10^{-7}$
Low (N*PS2:* 1–70)	43 (32.8)	32 (74.4)	5 (11.4)	6 (13.6)	
Intermediate (N*PS2:* 71–3,000)	44 (33.6)	9 (20.9)	19 (43.2)	16 (36.4)	
High (N*PS2:* 3,001–140,000)	44 (33.6)	2 (4.7)	20 (45.4)	22 (50.0)	
PR gene mRNA					$<10^{-7}$
Low (N*PR:* 1–17)	43 (32.8)	32 (74.4)	6 (13.6)	5 (11.4)	
Intermediate (N*PR:* 18–300)	44 (33.6)	10 (23.3)	23 (52.3)	11 (25.0)	
High (N*PR:* 301–12,000)	44 (33.6)	1 (2.3)	15 (34.1)	28 (63.6)	
ERβ gene mRNA					0.0013
Low (N*ERβ*: 1–16)	43 (32.8)	6 (14.0)	21 (47.7)	16 (36.4)	
Intermediate (N*ERβ*: 17–53)	44 (33.6)	13 (30.2)	13 (29.6)	18 (40.9)	
High (N*ERβ*: 54–550)	44 (33.6)	24 (55.8)	10 (22.7)	10 (22.7)	

[a] Relative gene target expression value, normalized both to an endogenous RNA control (*RPLP0* gene) and to a calibrator (breast tumor tissue sample that contained the smallest amount of target gene mRNA).
[b] χ^2 test (*ERα*-negative *versus ERα*-positive tumors; low *versus* intermediate/high gene mRNA expressed tumors).
[c] Number of patients (%).

linear dynamic range (at least six orders of magnitude) suitable for analyzing the expression of genes, such as *CGA*, that show large differences in the amount of mRNA molecules (*CGA* gene expression ranged from 0.3 to 16,500 times the level in normal breast tissues). Overexpression (>10 SD above the mean for normal breast tissues) of the *CGA* gene was observed in 44 (33.6%) of the 131 breast tumor RNA samples. Immunohistochemical studies confirmed hCGα protein overexpression and showed that it was strictly limited to breast tumor cells. Our results are in keeping with a report of hCGα protein production by breast tumor cells, as shown by immunohistochemistry analysis (12).

We sought to determine whether the observed overexpression of hCGα protein in breast cancer has to aim to associate with the hCGβ subunit to produce the heterodimeric hormone hCG, which could mediate biological activities through the luteinizing hormone/hCG receptor, which was recently shown to be expressed in breast cancer (13, 14). Although we observed large differences in the amount of *CGB* mRNA molecules (as high as 192 times the level in normal breast tissues), we found no link between *CGA* mRNA overexpression and *CGB* mRNA overexpression in our series of breast tumors (Table 3). We also observed no link between *CGA* overexpression and mRNA expression of the *LHB*, *FSHB*, and *TSHB* genes, which code for the other three β subunits capable of associating with the hCGα subunit to produce glycoprotein hormones (data not shown). The highest levels of *CGA* gene expression in our breast tumor series, which were close to those observed in placenta and were much higher than the amount of *CGB* mRNA in breast tumors ($>10^3$-fold lower that placental levels) suggest that different genetic (or epigenetic) mechanisms are responsible for *CGA* and *CGB* gene overexpression in breast tumors. Taken together, these results suggest that if the free hCGα subunit (and not the heterodimeric hormone hCG) is involved in breast tumorigenesis, it would act via a novel pathway not involving the luteinizing hormone/hCG receptor.

Table 5 *Subdivision of the 131 tumors according to mRNA CGA, ERα, PR, and PS2 status*

CGA status	*ERα* status	*PR* status	*PS2* status	No. of patients (%)
+[a]	+	+	+	35 (26.7)
+	+	+	−	6 (4.6)
+	+	−	+	2 (1.5)
+	+	−	−	1 (0.8)
+	−	+	+	0
+	−	+	−	0
+	−	−	+	0
+	−	−	−	0
−	+	+	+	33 (25.2)
−	+	+	−	3 (2.3)
−	+	−	+	7 (5.3)
−	+	−	−	1 (0.8)
−	−	+	+	8 (6.1)
−	−	+	−	3 (2.3)
−	−	−	+	3 (2.3)
−	−	−	−	29 (22.1)

[a] +, intermediate or high gene mRNA expression level; −, low gene mRNA expression level.

Table 6 *Relationship between mRNA CGA status and other molecular marker status in the 88 ERα-positive tumors*

	Total population	*CGA* normal expressed-ERα-positive tumors	*CGA* overexpressed-ERα-positive tumors	P^a
Total	88 (100.0)	44 (50.0)	44 (50.0)	
PS2 gene mRNA				NS[b]
Low	11 (12.5)[c]	4 (9.1)	7 (15.9)	
Intermediate	35 (39.8)	19 (43.2)	16 (36.4)	
High	42 (47.7)	21 (44.7)	21 (47.7)	
PR gene mRNA				NS
Low	11 (12.5)	8 (18.2)	3 (6.8)	
Intermediate	34 (38.6)	12 (27.3)	22 (50.0)	
High	43 (48.9)	24 (54.5)	19 (43.2)	
ERβ gene mRNA				NS
Low	37 (42.1)	15 (34.1)	22 (50.0)	
Intermediate	31 (35.2)	17 (38.6)	14 (31.8)	
High	20 (22.7)	12 (27.3)	8 (18.2)	
CCND1 gene mRNA				0.0024
Normal	52 (59.1)	33 (75.0)	19 (43.2)	
Overexpressed	36 (40.9)	11 (25.0)	25 (56.8)	

[a] χ^2 test.
[b] NS, not significant.
[c] Number of patients (%).

Little is known of the involvement of the hCGα subunit itself in carcinogenic processes. Rivera *et al.* (15) showed that an antisense sequence to the *CGA* gene inhibited the growth and reversed the transformed phenotype of human lung tumor cells. One possibility, in breast tissue, is that the hCGα subunit controls *PRL* (prolactin) gene expression, as in myometrial, decidual, and pituitary gland cells (16–18). Because *PRL* (prolactin) gene expression is altered in breast cancer (19), we tested this hypothesis. We found no link between *CGA* mRNA overexpression and *PRL* mRNA overexpression, ruling out a role of the *CGA* gene in *PRL* pathway dysregulation in breast tumors (data not shown).

The most important result of our study is that *CGA* gene overex-

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pression is perfectly linked to ER positivity in breast tumors. Indeed, *CGA* gene overexpression was observed in one-half of the ERα-positive tumors and in none of the ERα-negative tumors. We also found a weaker association between *CGA* gene overexpression and PR positivity and SBR histopathological grade I+II, which suggests that *CGA* is a marker of tumors with low aggressiveness. No relationship between ER positivity and *CGB* overexpression was observed in the same series. Our immunohistochemical data confirm that hCGα protein overexpression is strictly limited to ER-positive tumor cells. This makes *CGA* status an attractive candidate as a molecular marker, and one that may prove far more reliable than ER status alone, to predict endocrine responsiveness. Indeed, only one-half of all of the ER-positive patients respond favorably to antiestrogen treatment (1), and we observed *CGA* gene overexpression in one-half of the *ERα*-positive tumors in our breast tumor series. Moreover, we observed no link between *CGA* mRNA expression status and *PS2* or *PR* mRNA expression levels in the ERα-positive subgroup, which suggests that *CGA* activation requires ERα but involves a mechanism different from that of *PS2* and *PR*. Moreover, our results (summarized in Tables 4 and 5) suggest that the *CGA* gene may be a more reliable marker than *PS2* and *PR* for ERα functionality and, thus, for endocrine responsiveness. Indeed, several "*ERα*-negative" (low *ERα* mRNA expressed) tumors overexpressed *PS2* and/or *PR*, whereas none of the *ERα*-negative tumors overexpressed the *CGA* gene. Taken together, these findings suggest that the *CGA* marker provides new, independent information that, in conjunction with *ERα* status, may help to determine the likelihood that a given tumor will respond to endocrine therapy. We infer that the subpopulation of *CGA*-positive, *ERα*-positive tumors would correspond to those responsive to hormone therapy. Because endocrine therapy was rarely used 20 years ago, which is when our samples were obtained, we were unable to study the relationship between *CGA* gene overexpression and the response to endocrine therapy. However, among the 44 ERα-positive patients who received postoperative hormone therapy, 71.4% (15 of 21) of those who had *CGA* overexpression are relapse-free, compared with 43.5% (10 of 23) of those who had normal *CGA* expression ($P = 0.06$).

In addition to their clinical application, our results raise an important question concerning the mechanisms that lead to *CGA* overexpression in ERα-positive breast tumors. The structure of the *CGA* promoter has been extensively studied (reviewed in Ref. 20). Most studies have focused on *CGA* gene expression in placenta and pituitary tissues and suggest that the tissue-specific expression of the *CGA* gene is determined by combinations of several elements, some of which are common to all of the tissues, whereas others are specific to a given tissue. These studies identified a remarkable series of composite regulatory elements that interact with families of transcription factors that are still being characterized. These elements include a trophoblast-specific element (TSE) that binds TSEB and two CREs that bind CREBs. However, no consensus ERE was found in the 1638 bp of the cloned promoter region of the human *CGA* gene (GenBank accession no. AF109152). It is noteworthy that we observed no link between overexpression of the *CGA* gene and the *PS2* or *PR* genes, two ERα-inducible genes with ERE elements, which suggests that ERα activates *CGA* gene expression in breast tumors via a mechanism that does not involve an ERE element.

The most plausible hypothesis is that *CGA* gene activation by ERα protein occurs via the CRE element. Indeed, estrogen activates its receptor by inducing a conformational change in the hormone-binding domain (21), thereby creating a structural motif that binds to a number of transcriptional coactivators such as SRC-1 (steroid receptor coactivator), p300/CBP (CREB-binding protein), and P/CAF (p300/CBP-associated factor). Interestingly, p300/CBP can bind to CREB, which is known to bind to the CRE element, whereas P/CAF is an enzyme known to promote transcription by acetylating histones, thereby modifying the chromatin structure and facilitating gene expression.

In this respect it is noteworthy that the *CCND1* gene, the overexpression of which is also strongly linked to ER positivity in breast tumors, contains a CRE element but not an ERE element in its promoter. Sabbah *et al.* (22) recently showed that it is this CRE element that is involved in the activation of the *CCND1* gene by ERα. Interestingly, we observed a strong link between *CGA* mRNA overexpression and *CCND1* mRNA overexpression in our breast tumor series (Table 3) and especially in the *ERα*-positive subgroup (Table 6).

Keri *et al.* (23), using 1500 bp of the proximal 5′ flanking sequence of the human CGA gene in *in vitro* (cultured cells) or *in vivo* (transgenic mice) models, did not observe up-regulation by estrogen (or ERα) of reporter gene expression. We observed no CGA overexpression in the ERα-positive breast cell lines tested (MCF7, T-47D, ZR-75–1) either before or after estradiol treatment. Taken together, these results suggest that the mechanism that leads *in vivo* to CGA overexpression in human ERα-positive breast tumors involves several factors, including ERα and several uncharacterized transcriptional coactivators that are not all present in the classical *in vitro* and *in vivo* models used to date.

In conclusion, we have identified *CGA* as a major new ERα-responsive gene that should greatly contribute to our understanding of the role of the ER in breast cancer. Our results also make the *CGA* gene an attractive alternative molecular marker to *PR* and *PS2* as a refined predictor of endocrine responsiveness in breast cancer patients. We are currently conducting a large study of specific breast cancer patient phenotypes to test the practical value of *CGA* as a predictor of the response to hormone therapy. The *CGA* gene encodes a well-characterized secreted protein for which specific antibodies are already commercially available, thus facilitating the future application of this marker to the clinical management of breast cancer.

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