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Real-Time Reverse Transcription-PCR Assay for Future Management of *ERBB2*-based Clinical Applications

Ivan Bièche,[1,2] Peter Onody,[2] Ingrid Laurendeau,[1] Martine Olivi,[1] Dominique Vidaud,[1] Rosette Lidereau,[2] and Michel Vidaud[1*]

Background: **Gene amplification/overexpression of *ERBB2* (*HER2, neu*) is a major event in human breast tumorigenesis. *ERBB2*-based therapeutic agents and *ERBB2*-specific gene therapy are under development. These new perspectives call for a sensitive and accurate method to screen breast cancer patients for *ERBB2* alterations.**

Methods: **We have developed and validated a real-time quantitative reverse transcription (RT)-PCR assay, based on fluorescent TaqMan methodology, to quantify *ERBB2* gene expression at the mRNA level in breast tumors. This recently developed method of nucleic acid quantification in homogeneous solutions has the potential for a wide dynamic range, interlaboratory agreement, and high-throughput capacity without tedious post-PCR processing. The *ERBB2* mRNA signal was normalized to the signal for TATA box-binding protein mRNA.**

Results: **The dynamic range was >1000-fold. The relationship between C_t and log starting concentration was linear ($r^2 \geq 0.99$). The mean (SD) normalized expression of *ERBB2* in healthy breast tissue was 0.95 (0.37). Overexpression (>5 SD above mean for healthy breast) of the *ERBB2* gene was observed (at 3.2- to 135-fold) in 23 (17%) of 134 breast tumor RNA samples. As expected, *ERBB2* overexpression was present in all tumors with *ERBB2* gene amplification but was uncommon and at a low ratio (<5) in breast cancers without gene amplification.**

Conclusions: **This new simple, rapid, semi-automated assay is a major alternative to fluorescence in situ hybridization and immunochemistry for gene alteration analysis in human tumors and may be a powerful tool for large randomized, prospective cooperative group trials and to support future *ERBB2*-based biological and gene therapy approaches.**

The *ERBB2* gene (also known as *HER2* or *neu*) encodes a 185-kDa ($p185^{c\text{-}erbB2}$) transmembrane glycoprotein with intrinsic tyrosine kinase activity. $p185^{c\text{-}erbB2}$ has extensive sequence homology with the other members of the epidermal growth factor receptor family *(1)*. Amplification/overexpression of *ERBB2* is present in 15–25% of breast cancers and is associated with a poor prognosis *(2)*. Several recent studies have suggested that the poorer outcomes of such patients may be explained by a link between ERBB2 protein and responses and/or resistance to both chemotherapy *(3–5)* and endocrine therapy *(6, 7)*. ERBB2 expression status could thus be a useful marker to predict the response to cancer therapy. *ERBB2* is also an outstanding candidate therapeutic target gene *(8)*. Several *ERBB2*-based therapeutic strategies (anti-ERBB2 antibody, vaccines, and gene therapy) are being developed.

These promising new clinical perspectives call for a sensitive, accurate, and rapid method to screen breast cancer patients for amplification/overexpression of the *ERBB2* gene *(9)*. Such a method should be simple, widely applicable, and reproducible. The results should be subject to specific quality controls and provide the possibility for determining the CV. Whenever possible, values should be continuous rather than dichotomous because cutoff values are often arbitrary and suboptimal. Two principal methods are used: fluorescence in situ hybridization (FISH)[3] for the detection of gene amplification and

[1] Laboratoire de Génétique Moléculaire, Faculté des Sciences Pharmaceutiques et Biologiques de Paris, 4 Avenue de l'Observatoire, F-75006 Paris, France.

[2] Laboratoire d'Oncogénétique, Centre René Huguenin, F-92211 St-Cloud, France.

*Author for correspondence. Fax 33 1 44 07 17 54; e-mail mvidaud@teaser.fr.

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[3] Nonstandard abbreviations: FISH, fluorescence in situ hybridization; RT, reverse transcription; C_t, threshold cycle; TBP, TATA box-binding protein; and SBR, Scarff-Bloom-Richardson.

immunohistochemistry for the detection of gene expression at the protein level. Although these two techniques can measure alterations on an individual cell basis, they are complex and subject to considerable variations in the hands of different teams, posing problems for reproducibility and widespread use *(10–12)*. Moreover, FISH does not assess gene expression and cannot identify cases in which the gene product is overexpressed in the absence of gene amplification.

We developed a real-time quantitative reverse transcription (RT)-PCR assay based on TaqMan methodology to quantify *ERBB2* mRNA in homogeneous total RNA solutions obtained from tumor samples *(13)*. This real-time RT-PCR method has a major advantage over current quantitative PCR methods *(14)* as well as over FISH and immunohistochemistry, having a high-throughput capacity because no post-PCR manipulations are required. Moreover, the target input copy number is measured during the exponential phase of the reaction (when none of the reaction components is rate-limiting), leading to more accurate estimation of the sample concentration.

TaqMan methodology is based on the 5′ nuclease assay first described by Holland et al. *(15)*, which uses the 5′-3′ exonuclease activity of Taq polymerase to cleave a dual-labeled probe annealed to a target sequence during amplification. Release of a fluorogenic tag from the 5′ end of the probe is proportional to the target concentration and can be measured in "real time" during PCR amplification by a sequence detector (ABI PRISM 7700 Sequence Detection System; Perkin-Elmer Applied Biosystems).

We used this technique to measure *ERBB2* gene expression at the mRNA level in a series of 134 unilateral invasive primary breast tumors. We also determined whether overexpression of the *ERBB2* gene correlated with DNA amplification at the *ERBB2* locus, determined previously by a real-time PCR method *(16)*.

Materials and Methods

PATIENTS AND SAMPLES

We analyzed tissue from excised primary breast tumors of 134 women treated at the Centre 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 >60%. Immediately after surgery, the tumor samples were stored in liquid nitrogen until RNA extraction.

The patients (mean age, 58.3 years; range, 34–91 years) 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 8.2 years (range, 1.0–15.9 years). Forty-eight patients relapsed; the distribution of first relapse events was as follows: 14 local and/or regional recurrences, 30 metastases, and 4 both.

Table 1. Characteristics of the 134 patients and their relation to relapse-free survival.

		Relapse-free survival	
	Number of patients (%)	Number of events[a]	*P*[b]
Age			NS[c]
≤50	41 (30.6)	12	
>50	93 (69.4)	36	
Menopausal status			NS
Premenopausal	47 (35.1)	16	
Postmenopausal	87 (64.9)	32	
Histological grade[d]			NS
I	18 (14.4)	5	
II	60 (48.0)	25	
III	47 (37.6)	17	
Lymph node status			<0.05
Node-negative	50 (37.3)	10	
Node-positive	84 (62.7)	38	
ER status			NS
+ (≥10 fmol/mg)	89 (66.4)	34	
− (<10 fmol/mg)	45 (33.6)	14	
PR status			NS
+ (≥10 fmol/mg)	79 (59.0)	28	
− (<10 fmol/mg)	55 (41.0)	20	
Macroscopic tumor size			NS
≤30 mm	93 (73.2)	33	
>30 mm	34 (26.8)	13	

[a] First relapses (local and/or regional recurrences and/or metastases).
[b] Log-rank test.
[c] NS, not significant; ER, estrogen receptor; PR, progesterone receptor.
[d] SBR classification.

To help validate the kinetic quantitative RT-PCR method, we also analyzed five breast tumor cell lines obtained from the American Tissue Type Culture Collection (SK-BR-3, T-47D, BT-20, HBL-100, and MCF7).

Specimens of adjacent healthy breast tissue from 10 of the breast cancer patients and healthy breast tissue from 10 women undergoing cosmetic breast surgery were used as sources of normal RNA. Total RNA from a pool of six healthy human breast tissue samples was also purchased from Clontech.

EVALUATION OF "CLASSICAL" PROGNOSTIC FACTORS

The histological type and steroid-hormone receptor status of each tumor as well as the number of positive axillary nodes were established at the time of surgery. The malignancy of infiltrating carcinomas was scored according to the histoprognostic system of Bloom and Richardson *(17)*. Estrogen and progesterone receptor status was assayed as described by the European Organization for Research and Treatment of Cancer *(18)*, with a detection limit of 10 fmol/mg cytosolic protein.

REAL-TIME RT-PCR

Theoretical basis. The reactions are characterized by the point during cycling when amplification of the PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The parameter C_t (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The *ERBB2* target message in unknown samples is quantified by measuring C_t and by using a calibration curve to determine the starting target message quantity.

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 gene coding for the TATA box-binding protein (TBP, a component of the DNA-binding protein complex TFIID) as the endogenous RNA control, and each sample was normalized on the basis of its *TBP* content. We selected the *TBP* gene as an endogenous control because the prevalence of its transcripts is similar to that of the *ERBB2* target gene and because there are no known *TBP* retropseudogenes. (Retropseudogenes lead to coamplification of contaminating genomic DNA and thus interfere with RT-PCR despite the use of primers in separate exons). We therefore rejected the *β-actin, GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), and *HPRT* (hypoxanthine phosphoribosyltransferase) genes as endogenous controls because of the existence of corresponding retropseudogenes *(19, 20)*; we also rejected the human 18S rRNA gene, which is intronless, has no poly(A) tail, and has a very high abundance of transcripts; and the β_2-*microglobulin* gene, expression of which may be altered in some tumors *(21)*.

For each experimental sample, the amounts of the targets and endogenous reference were determined from the calibration curve. The target amount was then divided by the endogenous reference amount to obtain a normalized target value.

The relative gene target expression was also normalized to a healthy breast tissue sample (calibrator), or 1X sample. Each of the normalized target values was divided by the calibrator-normalized target value to generate the final relative expression.

Final results, expressed as *N*-fold differences in *ERBB2* gene expression relative to the *TBP* gene and the calibrator, termed "N_{ERBB2}", were determined as follows:

$$N_{ERBB2} = \frac{ERBB2_{SAMPLE}}{TBP_{SAMPLE}} / \frac{ERBB2_{CALIBRATOR}}{TBP_{CALIBRATOR}}$$

Primers, probes, and PCR consumables. Primers and probes for the *TBP* and *ERBB2* genes were chosen with the assistance of the computer programs Oligo 4.0 (National Biosciences) and Primer Express (Perkin-Elmer Applied Biosystems). We conducted BLASTN searches against dbEST and nr (the nonredundant set of GenBank, EMBL, and DDBJ 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 primer pairs for *ERBB2* were selected to be unique when compared with the sequences of the closely related *ERBB1 (EGFR)*, *ERBB3*, and *ERBB4* genes. The nucleotide sequences of the oligonucleotide hybridization probes and primers are shown in Table 2. The primers and probes are designated by the nucleotide position (relative to *TBP* GenBank accession no. X54993 and *ERBB2* GenBank accession no. M11730) corresponding to the 5′ position, followed by the letter U for upper (sense strand) or L for lower (antisense strand). To avoid amplification of contaminating genomic DNA, one of the two primers or the probe was placed at the junction between two exons or in a different exon. For example, the upper primer of *TBP* (706U) was placed in exon 5, the probe (745U) was placed at the junction between exon 5 and exon 6, and the lower primer (794L) was placed in exon 6.

Primers were purchased from Scandinavian Gene Synthesis AB and probes from Perkin-Elmer Applied Biosystems.

RNA extraction. Total RNA was extracted from breast specimens by the acid-phenol guanidinium method *(22)*. The quality of the RNA samples was determined by electrophoresis through denaturing agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under ultraviolet light.

Calibration curve. The calibration curve was constructed with fourfold serial dilutions of total RNA from healthy human breast tissue (cat. no. 64037-1; Clontech,) in mouse total RNA (cat. no. 64042-1; Clontech) with 1000, 250, 62.5, 15.6, and 3.9 ng of human normal breast total RNA. The

Table 2. Oligonucleotide primer and probe sequences used.

Gene	Oligonucleotide	Location	Sequence	PCR product size, bp
ERBB2	Upper primer	207U	5′-AGCCGCGAGCACCCAAGT-3′	147
	Lower primer	353L	5′-TTGGTGGGCAGGTAGGTGAGTT-3′	
	Probe	256U	5′-CCTGCCAGTCCCGAGACCCACCT-3′	
TBP	Upper primer	706U	5′-CACGAACCACGGCACTGATT-3′	89
	Lower primer	794L	5′-TTTTCTTGCTGCCAGTCTGGAC-3′	
	Probe	745U	5′-TGTGCACAGGAGCCAAGAGTGAAGA-3′	

series of diluted human total RNAs were aliquoted and stored at −80 °C until use.

cDNA synthesis. Reverse transcription of RNA was performed in a final volume of 20 μL containing 1× RT-PCR buffer (500 mmol/L each dNTP, 3 mmol/L $MgCl_2$, 75 mmol/L KCl, 50 mmol/L Tris-HCl, pH 8.3), 10 U of RNasin™ ribonuclease inhibitor (Promega), 10 mmol/L dithiothreitol, 50 U of Superscript II RNase H^- reverse transcriptase (Life Technologies), 1.5 mmol/L random hexamers (Pharmacia), and 1 μg of total RNA (calibration curve points and patient samples). 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 PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). For each PCR run, a master mixture was prepared on ice with 1× TaqMan buffer; 5 mmol/L $MgCl_2$; 200 mmol/L dATP, dCTP, and dGTP, and 400 mmol/L dUTP; 300 nmol/L each primer; 150 nmol/L probe; and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems). Ten microliters of each appropriately diluted reverse transcription sample (calibration curve points and patient samples) was added to 40 μL of the PCR master mixture. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min and 50 cycles at 95 °C for 15 s and 65 °C for 1 min.

Experiments were performed with duplicates for each data point. Each PCR run included the five points of the calibration curve (fourfold serially diluted human normal breast cDNAs), a no-template control, the calibrator cDNA, and 41 unknown patient cDNAs. The target gene mRNA copy value of the 41 patients was obtained in ~2 h with this assay format. All patient samples with a CV of the number of *ERBB2* mRNA copies >10% were retested.

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 factors 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 *(23)*, and the significance of differences between survival rates was ascertained using the log-rank test *(24)*.

Results

Calibration curve and dynamic range of real-time RT-PCR

The calibration curve was constructed from the total RNA extracted from healthy human breast tissues serially diluted fourfold in mouse total RNA (five points: 1000, 250, 62.5, 15.6, and 3.9 ng of human total RNA). The two primer pairs chosen to analyze the *TBP* and *ERBB2* genes do not amplify human genomic DNA or mouse cDNA (data not shown). The dynamic range was at least three orders of magnitude with samples containing as much as 50 ng or as little as 0.2 ng equivalent total cDNA. A strong linear relationship between the C_t and the log of the starting copy number was always demonstrated ($r^2 \geq 0.99$). The efficiency of the reaction (E), calculated by the formula: $E = 10^{1/|m|} - 1$, where m is the slope of calibration curve, was 90–100%.

ERBB2 mRNA in healthy breast tissues

To determine the cutoff for altered *ERBB2* gene expression at the RNA level in breast cancer tissue, the N_{ERBB2} value (ratio of *ERBB2* mRNA to *TBP* mRNA, calculated as described in *Materials and Methods*) was determined for 20 normal breast tissue RNAs. Because this value consistently fell between 0.5 and 1.7 (mean 0.95, 0.37), values of 3 (mean + 5 SD) or more were considered to represent overexpression of the *ERBB2* gene in tumor RNA samples.

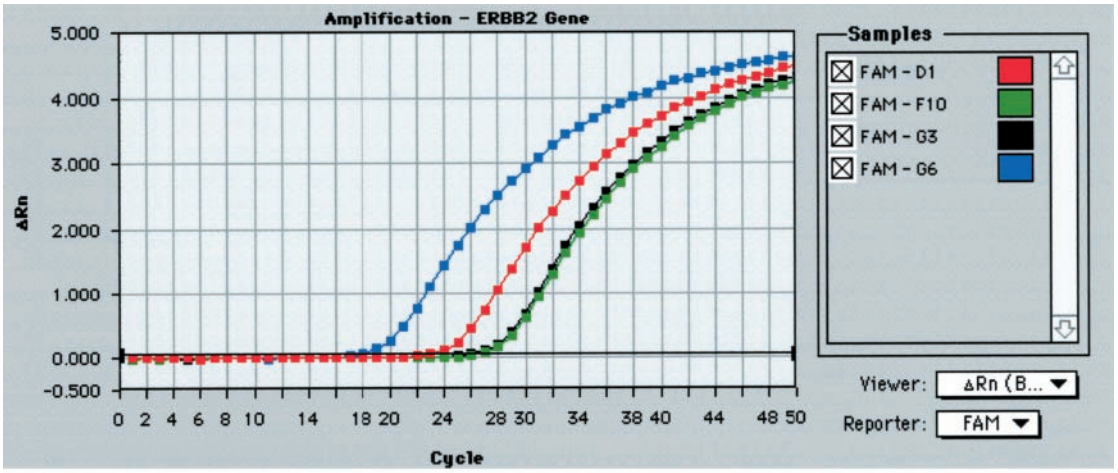
ERBB2 mRNA in tumor breast tissues

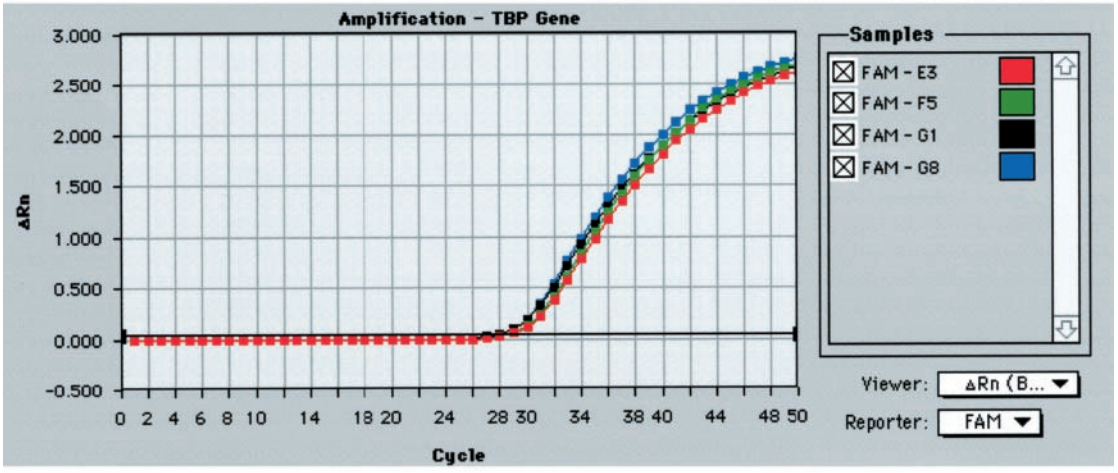
Among the 134 breast tumor RNA samples tested, 23 (17.2%) showed *ERBB2* overexpression. Major differences in *ERBB2* mRNA were observed (N_{ERBB2} from 3.2 to 135), with 13 (9.7%) tumors giving an expression level 3- to 10-fold, 6 (4.5%) tumors giving an expression level 10- to 30-fold, and 4 (3.0%) tumors giving an expression level >30 higher than that of healthy breast tissue. The strongest expression was 135-fold the basal level in tumor ERB98, which showed the greatest *ERBB2* gene amplification (15-fold) in our previously tested breast tumor DNA series *(16)*. Fig. 1 and Table 3 represent data on tumors in which the *ERBB2* gene was expressed 135-fold the basal value (sample ERB98), 11-fold the basal value (sample ERB37), and normally (sample ERB55). *ERBB2* expression was also investigated in five breast tumor lines, and only SK-BR-3 showed overexpression (N_{ERBB2} = 13). Lastly, among the 10 patients from whom both primary breast tumors and matched healthy breast tissue were investigated, two tumors (ERB114 and ERB67) showed clearly higher *ERBB2* expression in the tumor (N_{ERBB2} = 11.2 and 4.7, respectively) than in the healthy tissue (N_{ERBB2} = 0.9 and 1.2, respectively).

The *ERBB2* primer pair used in this study was placed in the 5′-terminal region of the *ERBB2* gene (extracellular domain of $p185^{c\text{-}erbB2}$), and total concordance was obtained with a second *ERBB2* primer pair in the 3′ region (intracytoplasmic domain of $p185^{c\text{-}erbB2}$; data not shown).

ERBB2 mRNA status and clinical and pathological factors

We sought links between the *ERBB2* mRNA status and standard clinical, pathological, and biological factors in breast cancer (Table 4). The statistically significant associations were between *ERBB2* gene overexpression and





		ERBB2		*TBP*	
	Sample	C_t	Copy number	C_t	Copy number
■	ERB98	17.6	653210	28.0	2200
■	ERB37	22.7	32518	28.8	1385
■	ERB55	26.1	4055	28.7	1504
■	CAL	25.8	4261	28.3	2052

Fig. 1. *ERBB2* and *TBP* mRNA measured by real-time RT-PCR in three breast tumor samples and the calibrator.

Samples: ERB98 (*G6*, *G8*, and *blue squares*), *ERB37* (*D1*, *E3*, and *red squares*), *ERB55* (*F10*, *F5*, and *green squares*), and *CAL* (*G3*, *G1*, and *black squares*) Given the C_t of each sample, the initial copy number is inferred from the calibration curve performed during the same experiment. Duplicates for each sample were performed, but the data for only one are shown here. The results are shown in Table 3.

Scarff-Bloom-Richardson (SBR) histopathological grade III ($P < 0.05$) and negative progesterone receptor status ($P < 0.001$). A trend toward a link between *ERBB2* gene overexpression and estrogen receptor negativity was also observed ($P = 0.09$).

Moreover, patients with tumors overexpressing *ERBB2* did not relapse more frequently (Table 4) and did not have significantly shorter relapse-free survival after surgery (log-rank test) compared with patients with tumors not overexpressing *ERBB2*.

Table 3. *ERBB2* mRNA status results.

	ERBB2			*TBP*				
Sample	**Copy number**	**Mean**	**SD**	**Copy number**	**Mean**	**SD**	***ERBB2/TBP* normalized**	**N_{ERBB2}[a]**
ERB98	653 210	644 431	12 415	2200	2274	104	283.4	134.9
	635 652			2347				
ERB37	32 518	31 473	1479	1385	1320	93	23.8	11.3
	30 427			1254				
ERB55	4055	4077	30	1504	1495	13	2.7	1.3
	4098			1485				
CAL	4261	4306	63	2052	2060	11	2.1	
	4350			2068				

[a] For each sample, the *ERBB2* mRNA copy number was divided by the *TBP* mRNA copy number to obtain a normalized *ERBB2/TBP* value, which was next divided by the normalized *ERBB2/TBP* value of the calibrator to obtain a final N_{ERBB2} value.

N_{ERBB2} and *ERBB2* amplification

Among the 134 tumors studied for *ERBB2* expression at the RNA level, 94 had previously been tested for *ERBB2* amplification, using the same TaqMan technology *(16)*. A near complete correlation between the results of real-time RT-PCR (*ERBB2* gene expression status) and real-time PCR (*ERBB2* gene amplification status) was obtained for tumors with high and moderate amounts of *ERBB2* messengers ($N_{ERBB2} > 5$). There were only three cases in which real-time RT-PCR showed *ERBB2* gene overexpression, whereas real-time PCR did not, but these three tumors had a low level of *ERBB2* gene overexpression ($N_{ERBB2} =$

Table 4. Relationship between mRNA *ERBB2* status and the standard clinical pathological and biological factors.

		Normal *ERBB2* mRNA	Overexpressed *ERBB2* mRNA	
	Total population (%)	**Number of patients (%)**	**Number of patients (%)**	***P*[a]**
Total	134 (100.0)	111 (82.8)	23 (17.2)	
Age, years				NS[b]
≤50	41 (30.6)	32 (28.8)	9 (39.1)	
>50	93 (69.4)	79 (71.2)	14 (60.9)	
Menopausal status				NS
Premenopausal	47 (35.1)	38 (34.2)	9 (39.1)	
Postmenopausal	87 (64.9)	73 (65.8)	14 (60.9)	
Histological grade[c]				<0.05
I	18 (14.4)	16 (15.7)	2 (8.7)	
II	60 (48.0)	53 (52.0)	7 (30.4)	
III	47 (37.6)	33 (32.3)	14 (60.9)	
Lymph node status				NS
Node-negative	50 (37.3)	41 (36.9)	9 (39.1)	
Node-positive	84 (62.7)	70 (63.1)	14 (60.9)	
ER status				NS
+ (≥10 fmol/mg)	89 (66.4)	78 (70.3)	12 (52.2)	
− (<10 fmol/mg)	45 (33.6)	33 (29.7)	11 (47.8)	
PR status				<0.001
+ (≥10 fmol/mg)	79 (59.0)	73 (65.8)	6 (26.1)	
− (<10 fmol/mg)	55 (41.0)	38 (34.2)	17 (73.9)	
Macroscopic tumor size				NS
≤30 mm	93 (73.2)	76 (73.1)	17 (73.9)	
>30 mm	34 (26.8)	28 (26.9)	6 (26.1)	
Relapses				NS
+	48 (35.8)	40 (36.0)	8 (34.8)	
−	86 (64.2)	71 (64.0)	15 (65.2)	

[a] χ^2 test.
[b] NS, not significant; ER, estrogen receptor; PR, progesterone receptor.
[c] SBR classification.

3.7, 4.5, and 4.8, respectively). We observed *ERBB2* gene overexpression in all tumors in which real-time PCR showed *ERBB2* gene amplification.

Discussion

Identification of altered gene function in cancer cells has led to the design of exciting new cancer treatments that specifically target these genetic changes. One promising example of this approach is the targeting of $p185^{c\text{-}erbB2}$ on the surface of *ERBB2*-overexpressing breast tumor cells, using monoclonal antibodies such as Herceptin, a recombinant humanized anti-$p185^{c\text{-}erbB2}$ monoclonal antibody that binds to the extracellular domain of the $p185^{c\text{-}erbB2}$ protein. Preliminary reports of Herceptin use, alone or in combination with chemotherapy, have shown its efficacy against breast cancers that express high levels of ERBB2 protein *(8, 25–27)*. Results with Herceptin are of interest because they provide a new therapeutic option for some patients in whom antiestrogen therapy (tamoxifen and raloxifen) is not effective *(8, 28)*. Indeed, *ERBB2*-overexpressing breast tumors are mainly estrogen receptor negative and thus unresponsive to antiestrogens *(29)*. In addition to monoclonal antibody-based therapies, several pharmaceutical companies are also developing gene therapy approaches against *ERBB2*. *ERBB2* antisense oligonucleotides down-regulate *ERBB2* expression and inhibit the growth and DNA synthesis of breast cancer cell lines *(30, 31)*. However, these new *ERBB2*-based clinical applications call for a simple, standardized, and rapid assay method *(9)*. *ERBB2* amplification/overexpression status has mainly been studied by means of two methods, FISH and immunohistochemistry, which assess specimens on an individual cell basis but have relatively little interlaboratory standardization and are not accurate enough to quantify the full range of alterations. Moreover, tumor biopsies arriving from different institutions and having undergone different fixation procedures may produce variations in the quality of FISH and immunohistochemistry. FISH also requires trained personnel and is time-consuming. Immunohistochemistry is subject to considerable variations in the hands of different teams because of alterations of target proteins during the procedure, different primary antibodies, and the criteria used to define positive staining. A study of several ERBB2 antibodies shows that their sensitivity varied from 22% to 79% *(10)*. These problems underscore the need for a new standardized *ERBB2* assay.

Gene expression status has also been studied at the RNA level by means of Northern blotting, but this method is not sensitive enough to detect low-level gene overexpression or accurate enough to quantify the full range of expression values. Northern blotting is also time-consuming, uses radioactive reagents, and requires large amounts of RNA, which means that it cannot be used routinely in clinical laboratories. An amplification step is required to determine the mRNA copy number of a given target gene from minimal quantities of tumor RNA (small early-stage tumors or cytopuncture specimens).

In this study, we validated an RT-PCR method recently developed for the quantification of gene expression *(13)*. The method, based on real-time analysis of PCR amplification and TaqMan methodology, has several advantages over other RT-PCR-based quantitative assays such as competitive quantitative RT-PCR *(14)*. The first advantage is that the real-time PCR method is performed in a closed-tube system, avoiding the risk of contamination by amplified products. The second advantage is the simplicity and rapidity of sample analysis because no post-PCR manipulations are required. The third advantage is that the system has a linear dynamic range of at least four orders of magnitude, meaning that samples do not need to contain equal starting amounts of RNA. This technique should, therefore, be suitable for analyzing small early-stage tumors, cytopuncture specimens, or formalin-fixed, paraffin-embedded tissues. The fourth, and perhaps most important, advantage is that real-time RT-PCR makes RNA quantification much more precise and reproducible because it is based on C_t values rather than end-point measurement of the amount of accumulated PCR product. Indeed, the ABI Prism 7700 Sequence Detection System enables C_t to be calculated when PCR amplification is still in the exponential phase and when none of the reaction components are rate-limiting. The within-run CV of the number of *ERBB2* mRNA copies for calibrator human RNA (five replicates) was always below 10%, and the between-assay CV in five different analytical runs was always below 20% (data not shown). Moreover, unlike competitive quantitative RT-PCR, real-time RT-PCR does not require an internal control. (The design and storage of internal controls and the validation of their amplification efficiency is painstaking).

The only potential disadvantage of real-time RT-PCR, like all other RT-PCR-based methods and solid matrix blotting techniques (Northern blots and dot blots), is that it cannot avoid dilution artifacts inherent in the extraction of RNA from tumor cells contained in heterogeneous tissue specimens. However, real-time RT-PCR has a fast turnaround time and requires small amounts of total RNA (5 ng/reaction in routine use), which means that it can be applied to several biopsy punches of very heterogeneous tumor samples.

Overall, the results of this study are in agreement with those reported in the literature: (*a*) The frequency (17%) of *ERBB2* alterations in our breast tumor series was similar to that reported by other teams *(11, 32)*. (*b*) The maximal *ERBB2* overexpression was 135-fold, in keeping with previous data obtained with other quantitative PCR-based assays *(33)*. (*c*) The *ERBB2* results obtained for the six breast tumor cell lines with real-time RT-PCR (i.e., SK-BR-3 overexpressed 13-fold) were in good agreement with previous data *(34)*. (*d*) The results of this study also confirm that *ERBB2* overexpression rarely occurs in breast cancer in the absence of *ERBB2* gene amplification *(11)*. (*e*)

We found an association between *ERBB2* gene overexpression and SBR histopathological grade III and steroid receptor negativity but not a poor prognosis, confirming that *ERBB2* is a marker of tumor aggressiveness rather than a major prognostic factor in human breast cancer [reviewed in Ref. *(35)*]. These latter findings must, however, be confirmed in a prospective and larger series of breast cancer patients. The relationship between *ERBB2* gene overexpression and the response and/or resistance to different therapies could not be studied in this retrospective series because the therapies used after surgery were highly variable.

We observed no prognostic importance of very low *ERBB2* expression in our series (only 3 relapses among the 10 (30%) patients with tumors showing an N_{ERBB2} value <0.5), as suggested by Koscielny *(36)*. Finally, we observed total concordance of our results when we used a primer pair placed in the 5′-terminal region of the *ERBB2* gene (extracellular domain of $p185^{c\text{-}erbB2}$) or in the 3′ region (intracytoplasmic domain of $p185^{c\text{-}erbB2}$), suggesting that the truncated *ERBB2* transcript (5′, 2.1 kb) that produces a secreted form of ERBB2 receptor containing only the extracellular ligand-binding domain *(37)* is not overexpressed alone without the full-length 4.6-kb *ERBB2* transcript.

In conclusion, we need today a reliable and reproducible technique for assessing *ERBB2* status with an established quality-control procedure to compare the *ERBB2* values between laboratories. Should we report gene amplification, protein expression, or mRNA levels? *ERBB2* status mainly has been studied with two methods, FISH and immunohistochemistry, that are not accurate enough to quantify (with SD determination) the full range of alterations. Here we describe a rapid, highly sensitive, high-throughput RT-PCR assay to determine *ERBB2* status. This method should be very useful for routine use in support of new *ERBB2*-based therapies for breast cancer.

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