Quantitation of *hTERT* Gene Expression in Sporadic Breast Tumors with a Real-Time Reverse Transcription-Polymerase Chain Reaction Assay[1]

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ABSTRACT

Recent observations support the notion that telomerase expression is essential for the formation of human tumor cells [W-C. Hahn *et al.*, Nature (Lond.), *400:* 464–468, 1999]. The expression pattern of *hTERT*, the human telomerase catalytic subunit gene, is a rate-limiting determinant of the enzymatic activity of human telomerase. We have developed a real-time quantitative RT-PCR assay based on TaqMan fluorescence methodology to quantify the full range of *hTERT* mRNA copy numbers. We validated the method on a series of 134 unilateral invasive primary breast cancer patients with known long-term outcome. Three-quarters of the breast tumors (75.4%; 101 of 134) were *hTERT* positive, *i.e.*, contained detectable and quantifiable *hTERT* mRNA. *hTERT*-positive patients had significantly shorter relapse-free survival ($P = 0.017$) after surgery compared with *hTERT*-negative patients. The prognostic significance of *hTERT* status persisted in Cox multivariate regression analysis. When we subdivided *hTERT*-positive patients ($n = 101$) into three equal groups (tumors showing small, intermediate, or high increase in *hTERT* mRNA content), we observed statistical (or a trend toward) links between high *hTERT* mRNA levels and Scarff-Bloom-Richardson histopathological grade III ($P = 0.066$), and negative estrogen ($P = 0.002$) and progesterone ($P = 0.048$) receptor status, and therefore with higher aggressiveness of breast tumors. High *hTERT* mRNA levels were also linked to *MYC* gene overexpression ($P = 0.007$). These findings show that the quantitative evaluation of *hTERT* mRNA can have important prognostic significance in human breast cancer. In addition, our simple, rapid, and semiautomated assay method is suitable for routine *hTERT* mRNA detection and quantification and will be a powerful tool in large, randomized, prospective, cooperative group trials and in the *hTERT*-based therapy project.

INTRODUCTION

Telomeres are structures located on the ends of eukaryotic chromosomes. In humans, they consist of several kilobases of a simple 5′-$(TTAGGG)_n$-3′ repeat. Telomeres protect the ends of chromosomes against degradation by exonucleases and ligases and against end-to-end fusion, rearrangements, and the loss of terminal DNA segments that occurs when linear DNA replicates (1). Telomeres in human somatic cells gradually shorten with each successive cell division, through replication-dependent sequence loss at DNA termini; this results in chromosome instability, leading to cellular senescence (2). A possible cause of human telomere shortening is repression of telomerase, a specialized ribonucleoprotein that consists of multiple protein subunits and a structural RNA component that contains a template sequence for the telomeric repeat (3). Telomerase activity is inactivated or repressed in the majority of normal somatic tissues but is activated in germ cells and in most malignant tumors. Telomerase reactivation may thus be a major step in human carcinogenesis (4).

Considerable interest is being focused on the potential use of telomerase-based assays as diagnostic and prognostic methods and for the development of telomerase-based therapies (5). To date, most studies have used the TRAP[3] to assay telomerase activity (6). In the TRAP assay, telomerase is extracted and allowed to synthesize extension products, which then serve as the templates for PCR amplification. However, the TRAP assay is time consuming, not accurate enough to quantify the full range of telomerase activity, and inappropriate to carry out retrospective studies of clinical outcome from standard archival material (7).

Recently, several components of human telomerase have been cloned, including the telomerase RNA component (*hTERC*; also termed *hTR*; Ref. 8) and the telomerase catalytic subunit (*hTERT*; also termed *hTRT*, *hTCS1*, or *hEST2*; Ref. 9). Telomerase activity correlates with the restricted expression pattern of the *hTERT* gene, whereas expression of the *hTERC*

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[3] The abbreviations used are: TRAP, telomeric repeat amplification protocol; RFS, relapse-free survival; RT-PCR, reverse transcription-PCR; SBR, Scarff-Bloom-Richardson.

gene is widespread (9). Ectopic *hTERT* expression is sufficient to confer enzymatic activity to telomerase-negative cells (10), suggesting that *hTERT* mRNA may serve as a surrogate index for telomerase activity. Ectopic *hTERT* expression in combination with two oncogenes (the SV40 large-T oncoprotein and an oncogenic allele of H-*ras*) results in direct tumorigenic conversion of normal human epithelial and fibroblast cells (11). Moreover, inhibition of *hTERT* results in telomere loss and limits the growth of human cancer cell lines *in vitro* and their tumorigenic capacity *in vivo* (12).

We have developed a real-time quantitative RT-PCR assay based on TaqMan methodology to quantify *hTERT* mRNA in homogeneous total RNA solutions prepared from tumor samples (13). This recently developed method is based on use of the 5′-3′ exonuclease activity of *Taq* polymerase to cleave a dual-labeled probe annealed to a target sequence during the extension phase of PCR. This method of nucleic acid quantification in homogeneous solution may become a reference in terms of its performance, accuracy, sensitivity, wide dynamic range, and high throughput capacity, and it also eliminates the need for tedious post-PCR processing. 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.

We used this technique to detect and to quantify *hTERT* gene expression in a series of 134 unilateral invasive primary breast tumor RNAs. We then compared *hTERT* gene expression with usual prognostic indicators and disease outcome.

In vitro studies suggest induction of telomerase activity by *MYC* overexpression (14, 15), and that both telomerase activity and Rb/CCND1/p16 pathway inactivation are necessary to immortalize human epithelial cells (16). In consequence, we also tested the possible link between *hTERT* expression levels and altered mRNA expression of *MYC*, *RB1*, and *CCND1* genes.

PATIENTS AND METHODS

Patients and Samples

We analyzed tissue from surgically removed primary breast tumors of 134 women followed 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 >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 8.9 years (range, 0.3–15.9 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).

To help validate the kinetic quantitative RT-PCR method, we also analyzed three breast tumor cell lines obtained from the American Tissue Type Culture Collection (SK-BR-3, BT-20, and MCF7). Ten specimens of adjacent normal breast tissue from breast cancers and normal breast tissue from 10 women undergoing cosmetic breast surgery were used to assess basal level of *hTERT* mRNA in normal breast tissue.

Table 1 Characteristics of the 134 patients and relation to RFS

	No. of patients	RFS No. of events (%)[a]	RFS P^b
Age			NS
≤50	41	12 (29.3)	
>50	93	36 (38.7)	
Menopausal status			NS
Premenopausal	47	16 (34.0)	
Postmenopausal	87	32 (36.8)	
Histological grade[c]			NS
I	18	5 (27.8)	
II	60	25 (41.7)	
III	47	17 (36.2)	
Lymph node status			0.022
Node-negative	50	10 (20.0)	
Node-positive	84	38 (45.2)	
ER status			NS
+ (≥10 fm/mg)	89	34 (38.2)	
− (<10 fm/mg)	45	14 (31.1)	
Macroscopic tumor size[d]			NS
≤30 mm	93	33 (35.5)	
>30 mm	34	13 (38.2)	
hTERT status			0.017
Negative	33	5 (15.2)	
Positive	101	43 (42.6)	

[a] Percentage of cases that had a relapse (local and/or regional recurrences, and/or metastases) in each subgroup of main clinical and pathological factors.
[b] Log-rank test. NS, not significant.
[c] SBR classification. Information available for 125 patients.
[d] Information available for 127 patients.

Real-Time RT-PCR

Theoretical Basis. Reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The larger the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The parameter 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 *hTERT* target message in unknown samples is quantified by measuring C_t and by using a standard 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 *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 breast tumor tissue sample that contained the smallest accurately quantifiable amount of *hTERT* mRNA. The calibrator thus indicates the limit of assay quantitation of the target which corresponds to a *hTERT* C_t value of 35. Each sample normalized *hTERT* value is divided

Table 2 Oligonucleotide primer and probe sequences used

Gene and oligonucleotide	Location	Sequence	PCR product size (pb)
hTERT			
Upper primer	2673U	5′-TGACACCTCACCTCACCCAC-3′	
Lower primer	2767L	5′-CACTGTCTTCCGCAAGTTCAC-3′	95
Probe	2711U	5′-ACCCTGGTCCGAGGTGTCCCTGAG-3′	
RPLP0			
Upper primer	95U	5′-GGCGACCTGGAAGTCCAACT-3′	
Lower primer	243L	5′-CCATCAGCACCACAGCCTTC-3′	149
Probe	205L	5′-ATCTGCTGCATCTGCTTGGAGCCCA-3′	

by the calibrator normalized *hTERT* value to give the final relative expression level.

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

$$N_{hTERT} = \frac{hTERT_{sample}}{RPLP0_{\text{sample}}} \Big/ \frac{\text{hTERT}_{\text{calibrator}}}{\text{RPLP0}_{\text{calibrator}}}$$

Primers, Probes, and PCR Consumables. Primers and probes for the *RPLP0* and *hTERT* 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 and nr (the non-redundant 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 nucleotide sequences of the oligonucleotide hybridization probes and primers are shown in Table 2. Primers and probes are designated by the nucleotide position (relative to *hTERT* GenBank accession number AF015950 and *RPLP0* GenBank accession number M17885) 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 was placed in a different exon. For example, the upper primer of *hTERT* (2673U) was placed in exon 10, whereas the probe (2711U) and the lower primer (2767 L) were placed in exon 11.

RNA Extraction. Total RNA was extracted from breast specimens by using the acid-phenol-guanidinium 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. Reverse transcription of RNA was done in a final volume of 20 μl containing 1× RT buffer (500 μM each deoxynucleotide triphosphate, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl, pH 8.3), 10 units of RNasin RNase inhibitor (Promega Corp., Madison, WI), 10 mM DTT, 50 units of Superscript II RNase H^- reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 1.5 μM 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.

Standard Curve Construction. The relative kinetic method was applied using a standard curve constructed with 4-fold serial dilutions of cDNA obtained from the MCF7 breast cell line known to strongly express the *hTERT* gene (17); the cDNA was obtained by reverse transcription from 1 μg of total RNA and 5-fold dilution in 1× RT buffer. The standard curve used for PCR is composed of 5 points (equivalent to 100, 25, 6.25, 1.6, and 0.4 ng of MCF7 total RNA).

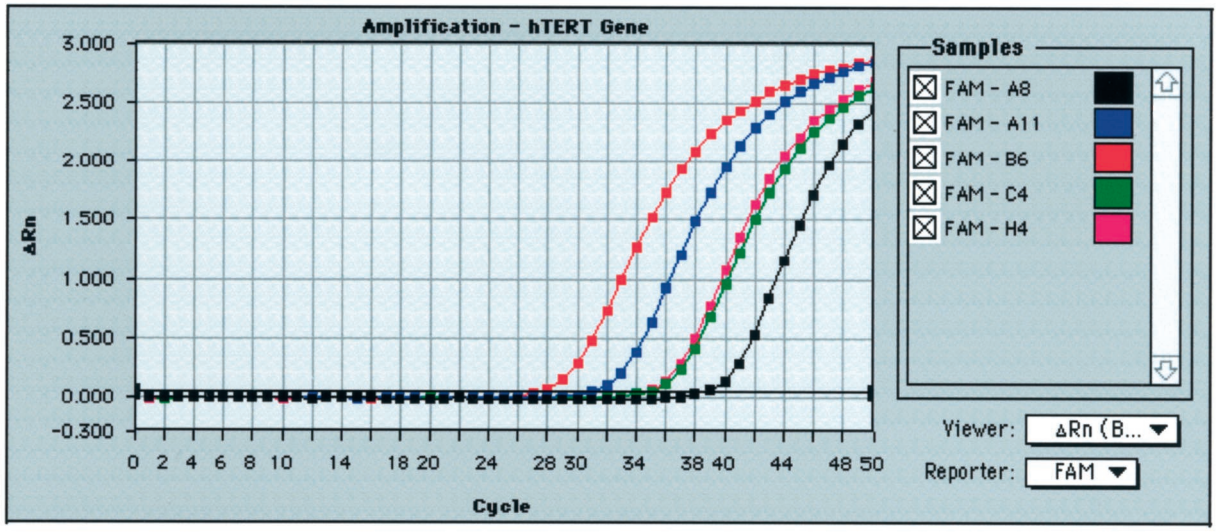
PCR Amplification. All PCR reactions were performed using a ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). For each PCR run, a master mix was prepared on ice with 1× TaqMan buffer, 5 mM $MgCl_2$, 200 μM dATP, dCTP, dGTP, and 400 μM dUTP, 300 nM each primer, 150 nM probe, and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems). Ten μl of each appropriately diluted RT samples (standard curve points and patients' samples) were added to 40 μl of the PCR master mix. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 65°C for 1 min.

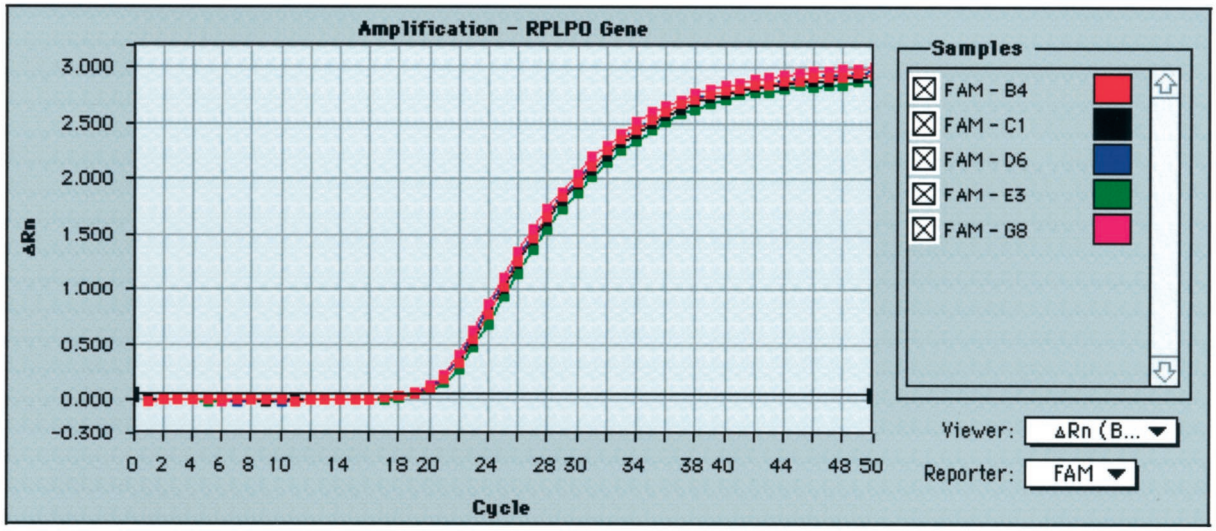
Experiments were performed with duplicates for each data point. Each PCR run included the five points of the standard curve (4-fold serially diluted MCF7 cell line cDNAs), a no-template control, the calibrator cDNA, and 41 unknown patient cDNAs. The target gene mRNA copy value of the 41 patients was obtained in ~2 h with this assay format.

Inclusion Criteria for *hTERT* Assay. Breast tissue samples were considered eligible for study when the *RPLP0* Ct value was ≤20, *i.e.*, suggesting an appropriate starting amount and quality of total RNA. All samples with a coefficient of variation for *RPLP0* and/or *hTERT* mRNA copy numbers >10% were also retested.

Statistical Analysis

RFS 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 >95% ($P < 0.05$). Survival distributions were estimated by the Kaplan-Meier method (18), and the significance of differences between survival rates was ascertained using the log-rank test. Multivariate analysis using Cox's proportional hazards model (19) was used to assess the independent contribution of each variable to RFS.





Sample	*hTERT* C_t	*RPLP0* C_t	N*hTERT*
TERT57	27.68	18.31	64.7
MCF7	31.16	18.57	8.2
TERT22	34.43	18.75	1.2
TERT91	38.84	18.72	negative
CAL	34.83	18.92	1

Fig. 1 hTERT and *RPLP0* mRNA amounts by real-time RT-PCR in the MCF7 cell line, three breast tumor samples, and the calibrator. MCF7 cell line (A11, D6, *blue squares*); tumor TERT57 (B6, B4, *red squares*); TERT22 (H4, G8, *pink squares*); TERT91 (A8, C1, *black squares*); CAL (C4, E3, *green squares*). The breast tumors are first divided into *hTERT*-positive samples (*hTERT* C_t, <35; *MCF7*, *TERT57*, and *TERT22*) and *hTERT*-negative samples (*hTERT* C_t, >35; *TERT91*). For the *hTERT*-positive samples, an N_{hTERT} is next determined, as described in "Patients and Methods." Briefly, the initial copy number of each sample is inferred from the C_t and by using the standard curve performed during the same experiment. The *hTERT* mRNA copy number is divided by the *RPLP0* mRNA copy number to obtain a normalized *hTERT/RPLP0* value, which is next divided by the normalized *hTERT/RPLP0* value of the calibrator to obtain a final N_{hTERT} value. Duplicates for each sample were performed, but the data for only one is shown here.

Table 3 Relationship between mRNA *hTERT* status (negative/positive) and the standard clinical pathological and biological factors

	Total population (%)	No. of patients (%)		p^a
		hTERT negative	*hTERT* positive	
Total	134 (100.0)	33 (24.6)	101 (75.4)	
Age				NS
≤50	41 (30.6)	11 (33.3)	30 (29.7)	
>50	93 (69.4)	22 (66.7)	71 (70.3)	
Menopausal status				NS
Premenopausal	47 (35.1)	12 (36.4)	35 (34.7)	
Postmenopausal	87 (64.9)	21 (63.6)	66 (65.3)	
Histological grade[b]				NS
I	18 (14.4)	5 (16.1)	13 (13.8)	
II	60 (48.0)	19 (61.3)	41 (43.7)	
III	47 (37.6)	7 (22.6)	40 (42.5)	
Lymph node status				NS
Node-negative	50 (37.3)	13 (39.4)	37 (36.6)	
Node-positive	84 (62.7)	20 (60.6)	64 (63.4)	
ER status				NS
+ (≥10 fmol/mg)	89 (66.4)	22 (66.7)	67 (66.3)	
−(<10 fmol/mg)	45 (33.6)	11 (33.3)	34 (33.7)	
PR status				NS
+ (≥10 fmol/mg)	79 (59.0)	22 (66.7)	57 (56.4)	
− (<10 fmol/mg)	55 (41.0)	11 (33.3)	44 (43.6)	
Macroscopic tumor size[c]				NS
≤30 mm	93 (73.2)	21 (70.0)	72 (74.8)	
>30 mm	34 (26.8)	9 (30.0)	25 (25.8)	
Relapses				0.004
+	48 (35.8)	5 (15.2)	43 (42.6)	
−	86 (64.2)	28 (84.8)	58 (57.4)	

[a] χ^2 test. NS, not significant.
[b] Scarff Bloom Richardson classification. Information available for 125 patients.
[c] Information available for 127 patients.

RESULTS

***hTERT* mRNA Detection in Normal Breast Tissues.** *hTERT* mRNA detection was assayed in 20 normal breast tissue RNAs. We observed no normal breast samples in which real-time PCR showed total absence of *hTERT* mRNA copies (*hTERT* C_t, 50). Indeed, a very faint positive signal was observed in all normal breast samples, with high *hTERT* C_t values. Because the *hTERT* C_t values consistently fell between 37 and 45, values of 35 or less were considered to represent overexpression of the *hTERT* gene in tumor RNA samples, which were thus scored *hTERT*-positive.

***hTERT* mRNA Levels in Breast Tumor Tissues.** Among the 134 breast tumor RNA samples tested, 33 (24.6%) were scored *hTERT* negative (*hTERT* C_t, >35) and 101 (75.4%) were scored *hTERT* positive (*hTERT* C_t, ≤35) with the possibility of N_{hTERT} value determination, calculated as described in "Patients and Methods." Among these 101 *hTERT*-positive tumors, major differences of N_{hTERT} values were observed, ranging from 1.0 to 64.7. *hTERT* gene expression was also investigated in three breast tumor cell lines (SK-BR-3, BT-20, and MCF7), which were scored *hTERT*-positive with N_{hTERT} values of 1.4 (BT-20), 3.6 (SK-BR-3), and 8.2 (MCF7). Fig. 1 gives data on one *hTERT*-negative tumor (TERT91), on MCF7 cell line (N_{hTERT} = 8.2) and on two *hTERT*-positive tumors with low (TERT22, N_{hTERT} = 1.2) and high (TERT57, N_{hTERT} = 64.7) *hTERT* mRNA levels.

The N_{hTERT} value was based on the amount of the *hTERT* target message relative to the *RPLP0* endogenous control to normalize the amount and quality of total RNA; similar results were obtained by using a second endogenous RNA control, the gene *TBP* coding for the TATA box-binding protein (a component of the DNA-binding protein complex TFIID; data not shown).

Correlation between Qualitative *hTERT* mRNA Status (*hTERT* negative/positive) and Clinical and Pathological Parameters. We sought links between qualitative *hTERT* mRNA status (*hTERT* negative/positive) and standard clinical, pathological, and biological factors in breast cancer (Table 3). *hTERT*-positive status was not significantly associated (χ2 test) with menopausal status or standard prognostic factors such as macroscopic tumor size, histopathological grade, or lymph node or steroid receptor status. Nevertheless, patients with *hTERT*-positive tumors had a higher rate of relapse [42.6% (43 of 101) *versus* 15.2% (5 of 33)] than those with a *hTERT*-negative tumors (P = 0.004; Table 3). *hTERT* positivity was associated with reduced RFS after surgery (log-rank test, P = 0.017; Table 1; Fig. 2). The outcome for the 101 patients with *hTERT*-positive tumors was significantly worse than that of the 33 patients with a *hTERT*-negative tumors in term of RFS [7-year RFS, 66.7% (57.1–76.3) *versus* 84.8% (72.6–97.1); 10-year RFS, 57.3% (46.3–68.2) *versus* 84.8% (72.6–97.1)].

Using a Cox proportional hazards model, we also assessed the prognostic value for RFS of parameters that were significant in univariate analysis, *i.e.*, lymph-node status and *hTERT* status

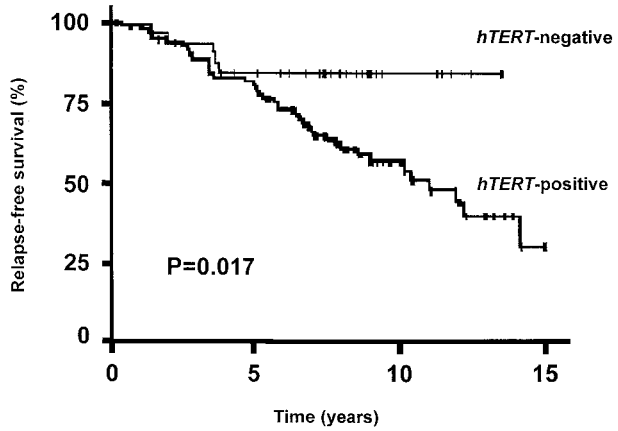


Fig. 2 RFS curves for patients with positive and negative hTERT tumors.

(Table 1). The prognostic significance of these two parameters persisted in Cox multivariate regression analysis (Table 4). Adjusted relative risk of these two parameters, taking into account menopausal status, macroscopic tumor size, histological grade, and steroid receptor status, did not change their prognostic significance for RFS (data not shown).

Correlation between Amount of *hTERT* mRNA and Clinical and Pathological Parameters. To better analyze the *hTERT* mRNA level as a quantitative variable, patients with *hTERT*-positive tumors ($n = 101$) were subdivided into three equal groups (34, 34, and 33 patients, respectively), with tumors with low (1.00–1.6), intermediate (1.6–3.5), and high (3.6–64.7) N_{hTERT} values. We observed statistical links between high *hTERT* mRNA levels and negative estrogen ($P = 0.002$) and progesterone ($P = 0.048$) receptor status (Table 5). A trend toward a link between high *hTERT* mRNA levels and SBR histopathological grade III was also observed ($P = 0.066$). The outcomes of the patients in the three groups did not differ (Table 5 and log-rank test not shown).

Relationship between *hTERT* mRNA Levels and *MYC, RB1,* and *CCND1* Expression Status. We tested the possible link between *hTERT* expression levels and altered mRNA expression of *MYC*, *RB1*, and *CCND1* genes, tested previously for same tumor RNA series.[4] We observed a statistical link between high *hTERT* mRNA levels and *MYC* overexpression ($P = 0.007$) but not between high *hTERT* mRNA expression and altered *RB1* or *CCND1* mRNA expression (Table 5).

DISCUSSION

Numerous published studies based on the TRAP assay have demonstrated that telomerase is activated in the vast majority of tumor types, including breast tumors, and also in certain normal tissues. Telomerase activity has been detected in 75–95% of breast tumor samples (20–24), and its up-regulation appears to be an early event in breast carcinogenesis (25).

[4] Unpublished data.

Table 4 Multivariate analysis of RFS

	RFS		
	Regression coefficient	Relative risk (95% CI)[a]	*P*
hTERT status (positive *vs.* negative)	1.07	2.92 (1.15–7.37)	0.024
Lymph node status (positive *vs.* negative)	0.79	2.21 (1.10–4.44)	0.027

[a] 95% confidence interval.

Studies that have correlated telomerase activity with clinical and pathological parameters have given conflicting results for traditional prognostic indicators, disease outcome, and telomerase activity (20–23). This suggests that telomerase activity may be influenced by parameters other than clinical/pathological features alone, such as sensitivity, telomerase inhibitors, and tissue viability with regard to the different TRAP protocols used. Overall, however, the results suggest that telomerase reactivation may be an important step in the progression of normal epithelial tissue to breast cancer.

Recently, the gene encoding the catalytic subunit of human telomerase (*hTERT*) has been cloned (9). Several studies have demonstrated that *hTERT* expression is a rate-limiting determinant of the enzymatic activity of human telomerase and that up-regulation of *hTERT* expression may play a critical role in human carcinogenesis (11). In a recent report based on an RNA *in situ* hybridization assay, Kolquist *et al.* (26) showed that *hTERT* expression appeared early during breast tumorigenesis *in vivo*, beginning in normal epithelial cells with proliferative potential and increasing gradually during the neoplastic process.

In this study, we validated a recently developed RT-PCR method for the quantification of *hTERT* expression (13). The method is based on real-time analysis of PCR amplification and TaqMan methodology and has several advantages over other RT-PCR-based quantitative methods, as well as over TRAP assay. The real-time PCR method does not require post-PCR sample handling, thereby avoiding problems related to carryover; it has a high sample throughput and possesses a wide dynamic range, meaning that samples do not have to contain equal starting amounts of total RNA. This technique should, therefore, be suitable for analyzing small early-stage tumors, fine needle aspiration specimens, or formalin-fixed, paraffin-embedded tissues. Real-time RT-PCR-based *hTERT* mRNA assay has also specific technical advantages over the TRAP assay: (*a*) because standard archival material (formalin-fixed, paraffin-embedded tissues) can be used to quantify *hTERT* mRNA by real-time RT-PCR, and retrospective studies of clinical outcome can be carried out; (*b*) real-time PCR reaction has endogenous control (*RPLP0* gene in this study) for each sample, whereas the controls for TRAP assay are from separate samples and reactions.

Finally, and above all, real-time PCR makes RNA quantitation much more precise and reproducible, being based on C_t values established in the early exponential phase of the PCR reaction (when none of the reagents is rate-limiting) rather than end point measurement of the amount of accumulated PCR

Table 5 Relationship between mRNA *hTERT* level and the standard clinical pathological and biological factors

	N_{hTERT}			
	Low	Intermediate	High	p^a
Total	34 (33.7)[b]	34 (33.7)	33 (32.6)	
Age				NS
≤50	9 (26.5)	12 (35.3)	9 (27.3)	
>50	25 (73.5)	22 (64.7)	24 (72.7)	
Menopausal status				NS
Premenopausal	12 (35.3)	12 (35.3)	11 (33.3)	
Postmenopausal	22 (64.7)	22 (64.7)	22 (66.7)	
Histological grade				0.066
I	6 (18.2)	6 (20.0)	1 (3.2)	
II	14 (42.4)	14 (46.7)	13 (41.9)	
III	13 (39.4)	10 (33.3)	17 (54.8)	
Lymph node status				NS
Node-negative	8 (23.5)	16 (47.0)	13 (39.4)	
Node-positive	26 (76.5)	18 (53.0)	20 (60.6)	
ER[c] status				0.002
+ (≥10 fmol/mg)	26 (76.5)	26 (76.5)	15 (45.5)	
− (<10 fmol/mg)	8 (23.5)	8 (23.5)	18 (54.5)	
PR status				0.048
+ (≥10 fmol/mg)	25 (73.5)	18 (53.0)	14 (42.4)	
− (<10 fmol/mg)	9 (26.5)	16 (47.0)	19 (57.6)	
Macroscopic tumor size				NS
≤30 mm	24 (70.6)	26 (83.9)	22 (68.7)	
<30 mm	10 (29.4)	5 (16.1)	10 (31.3)	
Relapses				NS
+	18 (52.9)	10 (29.4)	15 (45.5)	
−	16 (47.1)	24 (70.6)	18 (54.5)	
MYC mRNA				0.007
Normal	30 (88.2)	29 (85.3)	21 (63.6)	
Overexpressed	4 (11.8)	5 (14.7)	12 (36.4)	
RB1 mRNA				NS
Normal	24 (75.0)	27 (84.4)	22 (71.0)	
Underexpressed	8 (25.0)	5 (15.6)	9 (29.0)	
CCND1 mRNA				NS
Normal	28 (82.3)	23 (67.6)	28 (84.8)	
Overexpressed	6 (17.7)	11 (32.4)	5 (15.2)	

[a] χ^2 test:high N_{hTERT} value tumors *versus* low/intermediate N_{hTERT} value tumors. NS, not significant.
[b] Number of patients (percentage).
[c] ER, estrogen receptor; PR, progesterone receptor.

product. Real-time PCR has high intraassay and interassay reproducibility and gives statistical confidence values.

We validated the method on 20 normal breast tissue RNAs and on a large series ($n = 134$) of unilateral invasive primary breast tumor RNAs. *hTERT* mRNA was detected in 100% of breast tumor RNAs but also in all normal breast RNAs. These results reflect the higher sensibility of RT-PCR methods compared with the TRAP methods used, in agreement with Snijders *et al.* (27). These latter authors also showed that the presence of *hTERT* mRNA itself was not indicative of telomerase activity, but that a certain threshold level of *hTERT* mRNA is required for real telomerase activity. In our series, all of the normal breast tissue RNAs ($n = 20$) and 33 (24.6%) of the human breast tumor RNAs showed very low levels of *hTERT* mRNA that were only detectable but not quantifiable by means of the real-time quantitative RT-PCR assay. An increase in *hTERT* mRNA levels compared with the normal breast tissues was observed clearly in 75.4% of breast tumors. This frequency of *hTERT*-positive breast tumors is in agreement with data reported by other teams using the TRAP assay (20–24). Because the real-time quantitative RT-PCR assay is accurate enough to quantify the full range of expression values, the *hTERT*-positive group was subdivided into three equal subgroups, with tumors of low, intermediate, and high *hTERT* mRNA copy numbers. These additional cut points allowed to better study the possible correlations between *hTERT* gene expression levels and the usual prognostic indicators and disease outcome.

Overall, the results of this study agree with those reported in the literature: (*a*) We confirm, by quantitative evaluation of *hTERT* gene expression with a real-time RT-PCR assay, the association between telomerase activity in breast tumors and poor outcome reported by several previous studies based on the TRAP method (22, 23); (*b*) we observed associations between high *hTERT* mRNA levels and SBR histopathological grade III and steroid receptor negativity, in agreement with Roos *et al.* (22), who showed that high *hTERT* mRNA levels are associated with aggressiveness of breast tumors. These results suggest that tumor cells might be continuously selected for incrementally higher levels of telomerase activity as they proliferate and acquire genetic changes associated with invasive cancer (26). In this regard, Hiyama *et al.* (20) showed that only tumors with high telomerase activity exhibited altered telomere lengths (33%

of the breast tumors tested). This indicates that telomere alterations are linked to the multistep mutational events involved in tumor aggressiveness and occur a long time after reactivation of telomerase expression; (*c*) we observed a link between high *hTERT* expression levels and *MYC* gene overexpression. This *in vivo* study confirms the recently reported direct activation of *hTERT* transcription by c-myc transcriptor factor (15). Conversely, no correlation was observed between high *hTERT* expression levels and altered expression of the *RB1* and/or *CCND1* genes. This is in disagreement with data from Kiyono *et al.* (16), indicating that both telomerase activation and Rb/CCDN1/p16 pathway inactivation are required to immortalize primary epithelial cells.

In conclusion, this study points to a major role of the *hTERT* gene in breast tumorigenesis. In particular, we found evidence that *hTERT* mRNA status might serve as an exciting new prognostic tools in human breast cancer. These findings must now be confirmed in a larger series of breast cancer patients and in a large subpopulation of node-negative patients. Our rapid, highly sensitive, high-throughput RT-PCR-based *hTERT* mRNA assay should prove useful as a routine tool in *hTERT*-based clinical applications and therapeutic approaches to cancer.

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